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Effet des angiopoïétines sur la survie des neutrophiles

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Effet des angiopoïétines sur la survie des neutrophiles

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RÉSUMÉ

Nous avons identifié l'expression du récepteur des angiopoïétines, le récepteur Tie2, à la surface des neutrophiles humains. De plus, nous avons démontré qu'Ang1 et Ang2 induisent des activités pro-inflammatoires sur les neutrophiles, comme l'adhésion aux cellules endothéliales (CEs) et la synthèse du facteur d'activation plaquettaire (PAF). Puisque le PAF augmente la viabilité des neutrophiles et que les angiopoïétines modulent la survie des CEs, nous avons voulu évaluer l'effet des angiopoïétines sur la survie des neutrophiles. Des neutrophiles humains ont été isolés à partir du sang de donneurs sains en accord avec le comité d'éthique de l'Institut de cardiologie de Montréal. La viabilité des neutrophiles a été mesurée par cytométrie en flux à l'aide de marqueurs d'apoptose et de nécrose. Un traitement avec des témoins positifs, soit l'interleukine 8 (IL-8; 25 nM) ou le PAF (100 nM), a augmenté la survie basale des neutrophiles de 34 et 27%, respectivement. De plus, un traitement avec Ang1 (1 pM – 10 nM) a augmenté la survie des neutrophiles jusqu'à 35%, alors qu'Ang2 n'a eu aucun effet. La combinaison de l'IL-8 ou du PAF avec Ang1 (10 nM) a eu un effet additif sur la viabilité des neutrophiles et a augmenté la survie de 56 et 60%, respectivement. Un prétraitement avec des anticorps bloquants contre l'IL-8 a permis d'inhiber l'activité anti-apoptotique de l'IL-8 et d'Ang1 de 92 et 80%, respectivement. Ainsi, notre étude est la première à démontrer la capacité d'Ang1 à prolonger la viabilité des neutrophiles, qui est principalement causée par la relâche d'IL-8.

Mots clés : angiopoïétines, Ang1, Ang2, neutrophiles, survie, apoptose, cytokines, inflammation, PAF, IL-8

ABSTRACT

We reported the expression of angiopoietin receptor Tie2 on the surface of human neutrophils. In addition, we reported that Ang1 and Ang2 are both capable to promote pro-inflammatory activities in neutrophils, namely their adhesion onto endothelial cells (ECs) and platelet-activating factor (PAF) synthesis. PAF is known to promote pro-survival activity on neutrophils and since both angiopoietins can modulate ECs viability, we addressed whether Ang1 and/or Ang2 could modulate neutrophil viability. Human neutrophils were isolated from blood of healthy volunteers in accordance with the guidelines of the Montreal Heart Institute's ethical committee. Neutrophil viability was assessed by flow cytometry using apoptotic and necrotic markers. Treatment with anti-apoptotic mediators such as interleukin 8 (IL-8; 25 nM) and PAF (100 nM) increased neutrophil basal viability by 34 and 27%, respectively. In addition, treatment with Ang1 (1 pM – 10 nM) increased neutrophil viability by up to 35%, while Ang2 had no effect. Combination of IL-8 or PAF with Ang1 (10 nM) provided an additive effect on neutrophil viability and further increased viability by 56 and 60%, respectively. Pretreatment of the neutrophils with blocking anti-IL-8 antibodies inhibited the anti-apoptotic effect of IL-8 and Ang1 by 92 and 80%, respectively. In summary, our data are the first one to report Ang1 pro-survival activity on neutrophils, which is mainly driven through IL-8 release.

Key words: angiopoietins, Ang1, Ang2, neutrophils, viability, apoptosis, cytokines, inflammation, PAF, IL-8

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AA : acide arachidonique

$AACOCF_3$: arachidonoyl trifluoromethyl ketone

Ac : anticorps

Ang1 : angiopoïétine 1

Ang2 : angiopoïétine 2

BSA : albumine de sérum bovin

CDK : *cyclin-dependent kinases*

CE : cellule endothéliale

cIAP : *cellular inhibitor of apoptosis*

$cPLA_2$: phospholipase A_2 de type cytosolique

DMSO : diméthylsulfoxyde

EGF : *epidermal growth factor*

EIA : *enzyme immuno-assay* (essai immunologique enzymatique)

EPC : cellule endothéliale progénitrice

ERK : *extracellular signal regulated kinase*

FITC : fluorescein isothiocyanate

fMLP : formyl-Methionyl-Leucyl-Phenylalanine

GlyCAM : *glycosylation-dependent cell adhesion molecule*

GRO-α : *growth regulated oncogene-α*

ICAM : *intercellular adhesion molecule*

IL-8/CXCL8 : interleukine 8

IP-10 : *interferon γ-induced protein 10*

JNK : *c-Jun N-terminal kinase*

LPS : lipopolysaccharide

LTB_4 : leucotriène B_4

MAdCAM : *mucosal addressin cell adhesion molecule*

MAPK : *mitogen-activated protein kinase*

MIP-1 : *macrophage inflammatory protein 1*

MMP : métalloprotéase

MPO : myéloperoxidase

NF-κB : *nuclear factor κB*

NK : *natural killer*

NO : monoxyde d'azote

NRP : neuropiline

PAF : facteur d'activation plaquettaire

PBS : solution saline tamponnée au phosphate

PECAM : *platelet endothelial cell adhesion molecule*

PI3K : phosphatidylinositol-3-kinase

PKC : protéine kinase C

PlGF-1 : *placental growth factor*

PMN : polymorphonucléaire

PSGL-1 : *P-selectin glycoprotein ligand 1*

P.I. : iodure de propidium

RCPG : récepteur couplé aux protéines G

RNS : espèces réactives de l'azote

ROS : espèces réactives de l'oxygène

RPMI : *Roswell Park Memorial Institute medium*

SDRA : syndrome de détresse respiratoire aigu

SIRS : syndrome de réponse inflammatoire systémique

$sPLA_2$: phospholipase A_2 de type sécrétée

STAT-3 : *signal transducers and activators of transcription factor*-3

Tie : *tyrosine kinase with immunoglobulin and epidermal growth factor homology domains*

TLR : *toll-like receptor*

TNF-α : *tumor necrosis factor* α

TRAIL : *TNF-related apoptosis-inducing ligand*

VCAM : *vascular cell adhesion molecule*

VEGF : *vascular endothelial growth factor*

VEGFR : *vascular endothelial growth factor receptor*

XIAP : *x-linked inhibitor of apoptosis*

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1.0 INTRODUCTION

1.1 L'angiogenèse

L'angiogenèse est la formation de nouveaux vaisseaux sanguins à partir de vaisseaux préexistants [1]. Ce phénomène très complexe est finement régulé et sa participation est nécessaire à plusieurs processus physiologiques et pathologiques, parfois de façon bénéfique, comme dans le cas d'un infarctus, mais aussi de façon néfaste comme dans le cas d'un cancer tumoral [2]. L'angiogenèse étant à la fois essentielle physiologiquement et impliquée dans plusieurs pathologies, son fonctionnement et sa régulation ont été étudiés en profondeur pour ainsi tenter de moduler son action.

L'angiogenèse se produit en plusieurs étapes successives [1]. La première est une vasodilatation suivie d'une augmentation de la perméabilité vasculaire accompagnée d'une extravasation de certaines protéines plasmatiques dans le milieu extravasculaire. Cette augmentation de perméabilité est produite par le relâchement des jonctions interendothéliales et l'extravasation des protéines plasmatiques qui permettent de créer un échafaudage provisoire qui est nécessaire pour permettre la migration et l'ancrage extravasculaire des cellules endothéliales (CEs). Par la suite, les CEs relâchent complètement leurs jonctions interendothéliales et sécrètent des enzymes pour dégrader la matrice extracellulaire [1]. D'autres cellules sanguines, notamment les plaquettes et les leucocytes, participent aussi à cette étape en relâchant des protéases et des facteurs de

croissance [3, 4]. La rupture de la matrice extracellulaire permet ainsi la migration des CEs au site d'angiogenèse où elles formeront une structure tubulo-vasculaire, qui sera par la suite stabilisée par le recrutement de péricytes et de cellules musculaires lisses.

1.1.1 Implications physiologiques et pathologiques de l'angiogenèse

Au cours de l'embryogenèse, l'angiogenèse classique permet la formation du système vasculaire complet à partir d'un réseau sanguin primitif [2]. La formation de ce réseau primitif, aussi nommé vasculogenèse, se fait à partir des cellules endothéliales progénitrices. Par la suite, de la naissance jusqu'à l'âge adulte, l'angiogenèse classique participe à la croissance somatique en induisant le bourgeonnement de nouveaux vaisseaux à partir de vaisseaux existants. Chez l'adulte, l'angiogenèse physiologique a principalement trois fonctions, soit son implication dans le cycle menstruel et reproducteur de la femme, la réparation vasculaire suite à une blessure et la croissance musculaire suite à des efforts soutenus et fréquents. De plus, plusieurs stimuli, comme l'hypoxie et certains facteurs de croissance, peuvent induire une angiogenèse pathologique dans un grand nombre de maladies, dont le psoriasis, la rétinopathie, la polyarthrite rhumatoïde, l'athérosclérose et le cancer (tumeurs solides) [2]. À l'opposé, nous observons une régression vasculaire dans certaines pathologies comme la prééclampsie et les troubles d'ischémie-reperfusion où l'induction pharmacologique de l'angiogenèse pourrait diminuer les dommages causés par

l'hypoxie. Étant donné que l'angiogenèse est associée à plus de 70 maladies, ses mécanismes de régulation ont été étudiés en profondeur et la modulation de l'angiogenèse est vite devenue une cible thérapeutique de choix, particulièrement dans le traitement du cancer à tumeurs solides.

En effet, dans le cas du cancer tumoral, l'angiogenèse joue un rôle crucial puisqu'une tumeur solide ne peut pas dépasser la taille de 2-3 mm^3 sans avoir besoin d'un apport accru en oxygène et en nutriments [5]. L'hypoxie qui s'installe dans les cellules tumorales en mode prolifératif induit la synthèse de facteurs de croissance qui favorise l'angiogenèse et supporte ainsi l'expansion de la tumeur (Figure 1). De plus, plusieurs tumeurs expriment des gènes de certains facteurs de croissance, comme le VEGF, ce qui induit davantage le processus d'angiogenèse [6-10]. En plus de fournir oxygène et nutriments aux tumeurs, l'angiogenèse facilite l'accès des cellules tumorales à la circulation sanguine, l'implantation des cellules tumorales primaires dans des sites secondaires et le développement de métastases, affectant ainsi négativement le pronostic des patients. Ainsi, comme traitement anti-tumoral, plusieurs groupes de recherche ont tenté de bloquer l'angiogenèse à l'aide d'anticorps monoclonaux humanisés dirigés contre le VEGF et ce traitement est présentement utilisé en combinaison avec la chimiothérapie conventionnelle pour traiter le cancer du côlon, du sein et les carcinomes pulmonaires non à petites cellules [11-14]. Cette combinaison permet de réduire significativement l'angiogenèse et inhibe ainsi la croissance primaire et

métastatique des tumeurs. De plus, des études cliniques sont présentement en cours afin d'évaluer l'effet d'anticorps dirigés contre les récepteurs du VEGF [15].

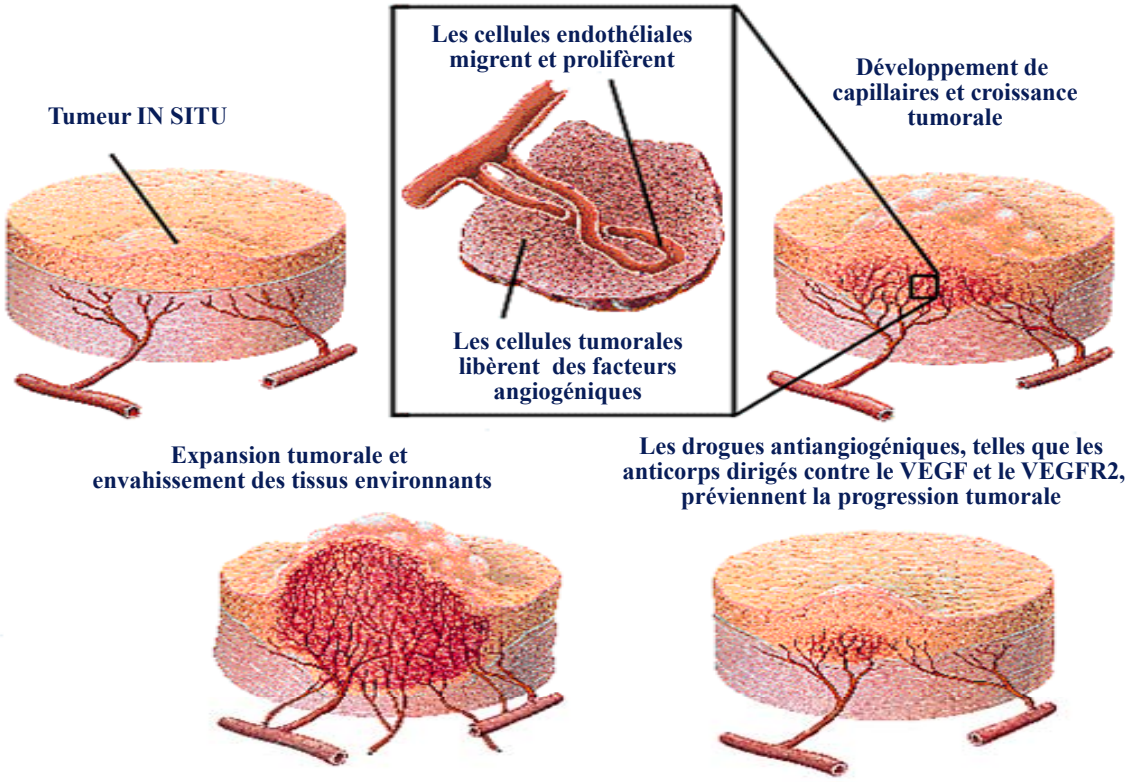

Figure 1. Rôle de l'angiogenèse et des thérapies antiangiogéniques dans l'expansion tumorale. En condition hypoxique, les cellules tumorales relâchent des facteurs angiogéniques qui stimulent la migration et la prolifération des CEs au site de la tumeur. Les nouveaux vaisseaux formés permettront l'expansion de la tumeur, alors que des thérapies antiangiogéniques inhiberont la formation de nouveaux vaisseaux et préviendront ainsi la croissance tumorale [16].

1.1.2 Régulation de l'angiogenèse par le VEGF

Le VEGF fut décrit pour la première fois en 1983 alors qu'un groupe de recherche avait isolé ce facteur capable d'induire la perméabilité vasculaire dans un modèle de cellules tumorales hépatiques [17]. Initialement nommé *vascular permeability factor*, il fut renommé VEGF à la fin des années 1980 après que Ferrara et Henzel aient constaté son effet mitogénique sur les CEs [18]. À ce jour, le VEGF est un des facteurs de croissance les mieux caractérisés et représente un facteur essentiel et prédominant dans le processus angiogénique [19]. Le VEGF est généralement employé pour décrire le VEGF-A, qui est composé de 5 isoformes différents, nommés VEGF-A_{121}, VEGF-A_{145}, VEGF-A_{165}, VEGF-A_{189} et VEGF-A_{206}, qui correspondent au nombre d'acides aminés qu'ils contiennent. Il existe également plusieurs protéines homologues au VEGF-A, soit le VEGF-B, le VEGF-C, le VEGF-D, le VEGF-E et le *placental growth factor* (PlGF-1) [20]. Le VEGF-A_{165} étant l'isotype le plus puissant et le plus abondant, nous nous concentrerons ici à décrire spécifiquement cet isoforme.

Une fois relâché, le VEGF peut lier l'héparine des protéoglycans héparanes sulfates membranaires avec un comportement propre à chaque isoforme, soit une liaison de 0 à 100%, et approximativement 50% du VEGF-A_{165} sécrété reste lié à l'héparine [21]. Le VEGF libre agit via l'activation de ces différents récepteurs, soient le VEGFR-1, le VEGFR-2 et le VEGFR-3, qui sont des récepteurs de type tyrosine kinase. Le VEGFR-1 lie principalement le VEGF-A, le VEGF-B ainsi que le

PlGF-1 [22] et est présent à la surface des monocytes, des macrophages, des ostéoclastes, des cellules musculaires, hématopoïétiques et endothéliales vasculaires [20, 23-25]. Le VEGFR-1 est aussi présent sous forme soluble et il possède la même affinité que les formes membranaires pour les isotypes du VEGF [26]. Le VEGFR-1 est principalement responsable du recrutement des cellules souches hématopoïétiques et de la migration des monocytes et des macrophages [27]. De plus, il participe à l'embryogenèse comme récepteur de clairance [28] et induit des activités biologiques par le biais de son hétérodimérisation avec le VEGFR-2. Notre laboratoire a d'ailleurs démontré que la synthèse de prostacycline induite par le VEGF-A_{165} est dépendante de cette hétérodimérisation [29] et qu'une thérapie génique par antisens dirigée contre le VEGFR-1 a permis d'inhiber l'effet angiogénique du VEGF-A_{165} de 85% [30]. De plus, le VEGFR-1 joue un rôle clé dans l'angiogenèse pathologique, plus particulièrement dans la polyarthrite rhumatoïde ainsi que le cancer [31]. Pour sa part, le VEGFR-2 lie le VEGF-A, le VEGF-C, le VEGF-D ainsi que le VEGF-E et est exprimé principalement à la surface des CEs vasculaires et lymphatiques. Bien que l'affinité du VEGFR-1 pour le VEGF-A_{165} soit approximativement 10 fois supérieure à celle du VEGFR-2, la majorité des activités biologiques du VEGF-A_{165} sont médiées par le VEGFR-2 [32]. En effet, son activation induit la prolifération, la migration, la survie et une augmentation de la perméabilité endothéliale [33]. En plus d'être le récepteur clé de l'angiogenèse, le VEGFR-2 est essentiel à l'embryogenèse et est impliqué dans plusieurs pathologies comme l'angiogenèse tumorale et la rétinopathie diabétique [34-36]. Finalement, le VEGFR-3 possède une affinité pour le VEGF-C ainsi que

le VEGF-D et est exprimé principalement sur les CEs lymphatiques [37]. Son expression est indispensable au cours de l'embryogenèse et sa principale fonction chez l'adulte est la lymphangiogenèse [38, 39]. Bien que ces trois récepteurs induisent l'activation subséquente de différentes voies de signalisation intracellulaires, ils stimulent tous de façon prédominante les voies PI3K-Akt et ERK1/2 [25, 38, 40-43].

En plus de lier ces trois récepteurs, le VEGF-A_{165} peut également lier la neuropiline 1 (NRP-1) [44]. La NRP-1 a initialement été décrite comme une protéine adaptatrice au VEGFR-2 qui permet d'augmenter la signalisation du VEGFR-2 induite par le VEGF-A_{165} [44-46]. Cependant, plus récemment, il a été observé que la NRP-1 peut aussi induire des effets biologiques indépendamment du VEGFR-2. En effet, des groupes de recherche ont démontré que la liaison du VEGF-A_{165} à la NRP-1 permet l'adhésion des CEs à la matrice extracellulaire et la migration des CEs via l'activation de la PI3K [47, 48]. Il existe également un autre type de neuropiline, la NRP-2, qui est aussi une protéine adaptatrice du VEGFR-2 et qui est impliquée principalement dans la lymphangiogenèse [49]. De plus, la NRP-1 et la NRP-2 sont impliquées dans plusieurs lignées de cellules tumorales. En effet, la NRP-1 favorise la croissance tumorale alors que la NRP-2 favorise le développement de métastases [50, 51].

Le VEGF-A_{165} induit directement l'angiogenèse dans plusieurs modèles [52, 53]. En effet, le VEGF-A_{165} induit la prolifération, la survie et la migration des CEs suivie de la formation de tubules-capillaires, qui sont les premières ébauches des futurs vaisseaux formés [25, 33]. De plus, le VEGF-A_{165} induit l'expression et la relâche endothéliale de protéases pour dégrader la matrice extracellulaire et de métalloprotéases (MMP) pour dégrader la membrane basale [54]. Bien qu'il soit surtout connu pour ces effets angiogéniques, le VEGF-A_{165} se différencie des autres facteurs de croissance par sa capacité à promouvoir aussi des effets pro-inflammatoires. En effet, le VEGF-A_{165} induit la synthèse endothéliale du monoxyde d'azote (NO), de la prostacycline (PGI_2) et du facteur d'activation plaquettaire (PAF), ce qui entraîne une augmentation de la vasodilatation et de la perméabilité vasculaire [46, 55]. De plus, le VEGF-A_{165} stimule l'expression et la translocation de molécules d'adhésion à la surface des CEs pour faciliter l'adhésion et le recrutement de leucocytes, plus particulièrement les neutrophiles et les monocytes [46, 56, 57]. Ces molécules d'adhésion sont la P-sélectine, la E-sélectine, l'*intercellular adhesion molecule* (ICAM-1) et le *vascular cell adhesion molecule* (VCAM-1).

Le VEGF étant surexprimé dans plusieurs lignées de cellules cancéreuses, des thérapies à l'aide d'anticorps anti-VEGF ont montré un certain potentiel à ralentir la prolifération et l'évasion des cellules tumorales, et bien que ces traitements soient bénéfiques, ils ne sont pas aussi efficaces qu'espérés [58]. Ces résultats semblent corréler avec le fait que d'autres facteurs de croissance puissent contribuer au

phénomène angiogénique, d'autant plus que le VEGF seul ne permet pas la maturation et la stabilisation des nouveaux vaisseaux formés [59, 60]. Une hypothèse a donc été émise sur l'implication de différents facteurs de croissance dans le processus d'angiogenèse. C'est ainsi qu'un autre groupe de facteurs de croissance, les angiopoïétines, a été identifié et évalué pour ses rôles dans le phénomène angiogénique.

1.2 Les angiopoïétines

Il existe 4 membres de la famille des angiopoïétines, soit Ang1, Ang2, Ang3 et Ang4 [61-63]. Les angiopoïétines partagent une structure similaire composée d'un court motif amino-terminal hydrophobe, suivie d'un domaine *coiled-coil* et d'un domaine carboxy-terminal de type fibrinogène (Figure 2) [61-63]. C'est ce dernier domaine qui est responsable de leur liaison à leur récepteur, soit le récepteur Tie2 (*tyrosine kinase with immunoglobulin and epidermal growth factor homology domains*) [64]. Ang1 et Ang2 sont les deux angiopoïétines les mieux caractérisées et sont impliquées principalement dans l'angiogenèse [61, 63, 65]. Ils partagent environ 60% de leur composition en acides aminés et ont une affinité similaire à lier le récepteur Tie2 [61, 63]. Ang3, qui est exprimée seulement chez la souris, et Ang4, qui est son orthologue humain, agissent également via le récepteur Tie2, et ce, en tant qu'agonistes au niveau des CEs [62, 66]. Le récepteur Tie2 appartient à la famille des récepteurs Tie qui comprend deux membres, soient les récepteurs Tie1 et Tie2, qui sont des récepteurs de type tyrosine kinase [67]. Ils possèdent une

structure similaire bien que les angiopoïétines lient seulement le récepteur Tie2 [67]. En effet, le récepteur Tie1 n'a aucun ligand connu à ce jour et est donc un récepteur orphelin. Cependant, il a été démontré qu'Ang1 et Ang4 ont la capacité d'activer Tie1 et il a été suggéré que Tie1 pourrait être activé via le récepteur Tie2, bien que les conséquences de l'activation de Tie1 soient encore mal comprises [65, 68-70]. Initialement, Ang1 a été identifiée comme un agoniste du récepteur Tie2 alors qu'Ang2 a été présentée comme un antagoniste de ce récepteur [61, 63]. Cette classification corrèle d'ailleurs avec les effets angiogéniques généralement opposés d'Ang1 et d'Ang2.

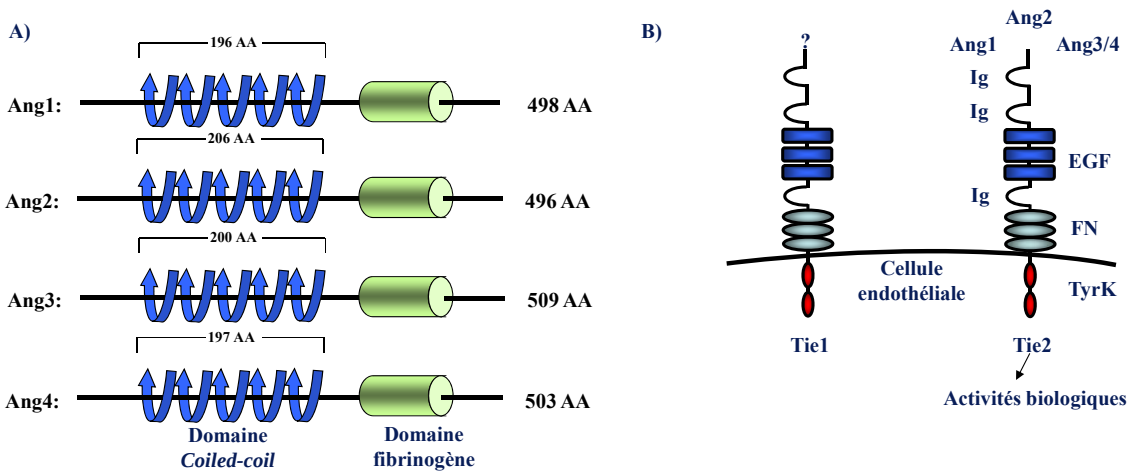

Figure 2. Structure des membres de la famille des angiopoïétines (A) et de leurs récepteurs, les récepteurs Tie1 et Tie2 (B). Les angiopoïétines sont composées principalement d'un court motif amino-terminal hydrophobe, suivies d'un domaine *coiled-coil* et d'un domaine carboxy-terminal de type fibrinogène. Les récepteurs Tie sont des récepteurs de type tyrosine-kinase et ont des structures similaires composées de trois domaines de type immunoglobuline, suivies de trois

domaines de type EGF (*epidermal growth factor)*, de trois répétitions de fibronectine de type 3 et de deux domaines intracellulaires de type tyrosine kinase [67] (adaptée d'après [71]).

En effet, au cours de l'embryogenèse, l'expression d'Ang1 est essentielle et contribue de façon très importante à la formation d'un réseau vasculaire mature [72]. Le modèle de souris *knock-out* pour Ang1 entraîne donc une létalité embryonnaire caractérisée par une vascularisation insuffisante et immature [72]. Ces caractéristiques peuvent aussi être observées dans les modèles murins *knock-out* du VEGFR-2 et du récepteur Tie2 [35, 73, 74]. En effet, la létalité embryonnaire engendrée par tous ces modèles se produit approximativement à la même période, soit entre le 8^{ième} et le 13^{ième} jour [35, 72-74]. À l'opposé, une surexpression d'Ang1 entraîne une augmentation de la taille et de la densité de la vasculature accompagnée d'une diminution de l'hyperperméabilité induite par la réponse inflammatoire, suggérant qu'Ang1 aurait aussi des propriétés anti-inflammatoires pour assurer la stabilité et l'intégrité vasculaire [75, 76]. Pour ce qui est d'Ang2, le modèle murin de *knock-out* est viable bien que la majorité des nouveau-nés décèdent avant le 14^{ième} jour post-natal suite à des malformations du système lymphatique, où Ang2 agit comme agoniste du récepteur Tie2 et favorise ainsi la croissance et le développement de ce système [77]. De plus, la surexpression d'Ang2 entraîne une létalité embryonnaire caractérisée par des malformations cardiaques et vasculaires [63]. Ce phénotype, qui est similaire à celui observé dans le modèle murin de *knock-out* d'Ang1 confirme qu'Ang1 et

Ang2 ont des effets opposés sur l'angiogenèse classique embryonnaire, alors qu'au niveau du système lymphatique Ang2 semble jouer un rôle semblable à celui d'Ang1 dans la vascularisation.

Chez l'adulte, l'expression d'Ang1 est constitutive et se retrouve dans plusieurs tissus comme l'utérus, les ovaires, le système nerveux central, les intestins, le pancréas et les muscles squelettiques [63]. Au niveau de la vasculature, Ang1 n'est pas exprimée par les CEs mais plutôt par les péricytes et les cellules musculaires lisses [61]. Notre laboratoire a d'ailleurs démontré la présence d'Ang1 dans les neutrophiles, où elle est localisée dans le cytosol [78]. Certains stimuli, comme l'hypoxie et le VEGF, stimulent l'expression d'Ang1 et sa relâche subséquente permet d'activer de façon paracrine le récepteur Tie2 qui se trouve principalement à la surface des CEs [79].

À l'opposé, chez l'adulte, Ang2 est très peu exprimée constitutivement mais son expression est rapidement augmentée lors de l'angiogenèse physiologique, que se soit au niveau du cycle reproductif de la femme ou encore au niveau d'une réparation vasculaire [80, 81]. Ang2 est exprimée principalement dans les CEs où elle est emmagasinée dans les corps de Weibel-Palade [82]. Elle induit donc ses activités biologiques par effet autocrine. De plus, son expression est augmentée par les mêmes stimuli que pour Ang1, soient principalement l'hypoxie et le VEGF [83, 84].

1.2.1 Régulation de l'angiogenèse par Ang1 et Ang2

Au cours de l'angiogenèse, Ang1 agit tout d'abord comme agent chimiotactique pour les CEs [85]. Elle permet ainsi le bourgeonnement des CEs et leur formation subséquente en tubules-capillaires *in vitro* [86, 87]. De plus, Ang1 induit la maturation des néo-vaisseaux en stabilisant les jonctions interendothéliales ainsi qu'en favorisant le recrutement de cellules péri-endothéliales et la survie des CEs [72, 88-90]. Ces propriétés anti-inflammatoires, particulièrement la diminution de la perméabilité vasculaire, sont d'ailleurs responsables de l'effet bénéfique d'Ang1 dans certaines maladies telles que l'athérosclérose et la septicémie [75, 91, 92]. À l'opposé d'Ang1, Ang2 a été initialement identifiée comme un antagoniste du récepteur Tie2 qui induit l'augmentation de la perméabilité endothéliale et favorise l'apoptose des CEs [63, 93]. Ainsi, Ang2 participe à l'initiation du processus angiogénique où elle est essentielle à la déstabilisation des vaisseaux sanguins existants pour permettre le bourgeonnement des nouveaux vaisseaux, alors qu'à l'opposé, Ang1 participe à finaliser le processus angiogénique étant donné que la maturation et la stabilisation des nouveaux vaisseaux sanguins sont essentielles pour maintenir les néo-vaisseaux fonctionnels à long terme [94]. Cependant, plus récemment, certaines études ont démontré qu'Ang2 peut aussi agir comme agoniste du récepteur Tie2 et ainsi induire la phosphorylation de ce récepteur [95-97]. De plus, Ang2 est capable d'induire plusieurs activités agonistes ; elle est un agent chimiotactique pour les CEs, elle induit la formation de tubes dans un modèle murin cérébral et certaines études suggèrent qu'elle a la capacité d'augmenter la viabilité des CEs, bien que d'autres groupes de recherche suggèrent le contraire

[95-101]. De plus, seule Ang2 est capable de favoriser la migration des cellules endothéliales progénitrices (EPC) et leur adhésion aux CEs, ce qui suggère qu'elle est essentielle au recrutement de ces cellules [102]. Les angiopoïétines ayant plusieurs fonctions angiogéniques communes avec le VEGF, l'hypothèse a été émise qu'Ang1 et Ang2 puissent également induire des activités biologiques pro-inflammatoires sur les CEs.

1.2.2 Effets pro-inflammatoires des angiopoïétines sur les CEs

Bien qu'Ang1 possède des propriétés anti-inflammatoires dans plusieurs modèles d'angiogenèse, plusieurs groupes d'études ont récemment démontré que les deux angiopoïétines ont la capacité d'induire certains effets pro-inflammatoires dans les CEs, et ce avec une puissance similaire. En effet, Ang1 et Ang2 ont la capacité d'augmenter la survie des CEs, bien que l'effet d'Ang2 sur la modulation de la viabilité des CEs semble dépendant des conditions expérimentales [96, 98, 100, 101, 103, 104]. En effet, différents groupes ont observé qu'Ang2 augmente la survie des CEs via l'activation de la voie PI3K [96, 100], alors que d'autres études ont démontré qu'Ang2 induit une activité pro-apoptotique sur les CEs et ont suggéré que l'effet d'Ang2 soit causé par la déstabilisation des jonctions de VE-cadhérines [105] ou par l'activation de la caspase-3 et de la relâche de Bax [101]. De plus, nous avons démontré que les deux angiopoïétines induisent la synthèse endothéliale du PAF [106], qui est un puissant agent pro-inflammatoire, en plus d'induire la translocation de la P-sélectine [107, 108], qui est une molécule

d'adhésion qui favorise le recrutement des neutrophiles et leur migration subséquente. Les deux angiopoïétines ont aussi la capacité de potentialiser l'effet pro-inflammatoire de cytokines et de facteurs de croissance. En effet, Ang2 a la capacité de sensibiliser les CEs au TNF-α et d'ainsi favoriser l'adhésion des leucocytes aux CEs [109]. De plus, les deux angiopoïétines potentialisent l'effet du VEGF dans un modèle murin de néovascularisation post-natale de la cornée [110]. En effet, dans ce modèle, la combinaison d'Ang1 et du VEGF permet la formation de vaisseaux sanguins plus denses avec des diamètres luminaux plus grands, alors que la combinaison d'Ang2 et du VEGF permet la formation de vaisseaux plus longs [110].

1.2.3 Effets pro-inflammatoires des angiopoïétines sur les neutrophiles

Après avoir constaté que les angiopoïétines avaient la capacité d'induire la translocation de la P-sélectine, nous avons évalué leurs effets sur l'adhésion des neutrophiles aux CEs [107, 108]. Nous avons observé qu'Ang1 et Ang2 avaient une capacité similaire à augmenter l'adhésion des neutrophiles et qu'une combinaison des deux angiopoïétines avait un effet additif sur l'adhésion [107]. Étant donné que la combinaison d'Ang1 et d'Ang2 n'avait aucun effet additif sur la translocation de la P-sélectine, nous avons émis l'hypothèse que les angiopoïétines puissent agir directement sur les neutrophiles. Nous avons donc démontré, pour la première fois, la présence du récepteur Tie2 à la surface des neutrophiles [107]. De plus, nous et d'autres groupes de recherche ont démontré qu'Ang1 et Ang2

promouvoient plusieurs effets pro-inflammatoires sur les neutrophiles [107, 111, 112]. En effet, les angiopoïétines induisent la synthèse de PAF et l'activation du CD11b/CD18 (β_2-intégrine), qui est une molécule d'adhésion qui favorise la liaison des neutrophiles aux CEs [107]. Les angiopoïétines ont également la capacité d'induire directement la migration des neutrophiles et de potentialiser l'effet de l'IL-8 sur cette activité biologique [112].

1.2.4 Mécanismes intracellulaires impliqués dans la signalisation des angiopoïétines

Dans les CEs, nous avons démontré qu'Ang1 et Ang2 ont la capacité d'activer différentes voies de signalisation, soient les voies p38 et p42/44 MAPK ainsi que la voie PI3K/Akt [106]. Plus précisément, la synthèse endothéliale du PAF induite par Ang1 et Ang2 est dépendante des voies p38 et p42/44 MAPK, de la voie PI3K et de l'enzyme $sPLA_2$-V [106], alors que la translocation de la P-sélectine est calcium-dépendante et est régulée par l'activation de la PLCγ [108]. De plus, l'activation de la voie PI3K est responsable de l'augmentation de la viabilité des CEs induite par Ang1 et Ang2 [96, 100, 103, 104]. Finalement, pour ce qui est des neutrophiles, nous avons démontré que les deux angiopoïétines stimulaient de façon similaire la voie PI3K et que cette activation est responsable de la capacité d'Ang1 et Ang2 à induire la migration des neutrophiles [112].

1.2.5 Implications pathologiques des angiopoïétines

Les angiopoïétines sont associées à plusieurs pathologies, plus particulièrement le cancer tumoral et l'arthrite rhumatoïde [113, 114]. En effet, les 2 angiopoïétines sont surexprimées dans plusieurs lignées de cellules tumorales. De plus, les concentrations plasmatiques d'Ang1 et d'Ang2 sont généralement augmentées en présence d'une tumeur et nous observons une modification du ratio Ang2 par rapport à Ang1 en faveur de l'Ang2 [113]. Ang1 et Ang2 contribuent au processus angiogénique et facilitent ainsi la prolifération des cellules tumorales. De plus, les angiopoïétines favorisent la formation de métastases, ce qui en fait une cible de choix comme agent anti-tumoral. Ainsi, des études cliniques sont présentement en cours pour évaluer l'effet d'anticorps humanisés dirigés contre Ang2 utilisés en combinaison avec des anticorps dirigés contre le VEGF et la chimiothérapie conventionnelle [113].

En conditions physiologiques, les articulations sont protégées par la membrane synoviale et la substance lubrifiante qu'elle sécrète, la synovie. Dans le cas de l'arthrite rhumatoïde, qui est une pathologie auto-immune, une inflammation et une hypervascularisation s'installent au niveau des articulations, plus précisément au niveau de la membrane synoviale, ce qui mènent à une prolifération de fibroblastes et à une relâche d'enzymes catalytiques, qui, à leurs tours, mènent à un épaississement de la membrane synoviale, une dégradation du cartilage et des extrémités des os ainsi qu'une perte de fonction importante [115]. Dans cette

pathologie, on retrouve une forte expression d'Ang1, d'Ang2 et des récepteurs des angiopoïétines, soit les récepteurs Tie1 et Tie2, dans les vaisseaux sanguins de la membrane synoviale. De plus, ces molécules pro-angiogéniques sont aussi fortement exprimées dans la paroi synoviale et dans les cellules stromales. Il a aussi été démontré qu'Ang1 et Ang2 induisent la migration *in vitro* des fibroblastes synoviaux de patients atteints d'arthrite rhumatoïde [114]. Finalement, le TNF-α, qui est une cytokine fortement exprimée dans l'arthrite rhumatoïde, stimule l'expression d'Ang1 dans les synoviocytes [116].

1.3 L'inflammation

L'inflammation est un processus essentiel pour initier la réponse immunitaire, qu'elle soit innée ou acquise. En effet, suite à une lésion ou une infection, plusieurs substances spécifiques se retrouvent au site de la lésion, que se soit des molécules bactériennes, comme des lipopolysaccharides (LPS), ou encore des molécules sécrétées par des cellules endommagées, apoptotiques ou nécrotiques, comme les prostaglandines, les leucotriènes, le PAF et les interleukines. Ces molécules induisent tout d'abord une vasodilatation qui entraîne un œdème du site lésé, puis elles agissent sur les CEs pour induire la translocation de molécules d'adhésion qui favorisera la migration de cellules inflammatoires, en plus d'agir comme agents chimiotactiques pour attirer les leucocytes au site de la lésion. Les premières cellules recrutées sont les neutrophiles, qui par le phénomène de diapédèse, migreront au site lésé et relâcheront ensuite une multitude d'enzymes, de cytokines

et de chimiokines qui entraîneront une dégradation du milieu extracellulaire, une toxicité cellulaire et bactérienne, une intensification de l'état inflammatoire et un recrutement accru de leucocytes. L'arrivée des monocytes, des éosinophiles, des basophiles et des lymphocytes intensifient l'état inflammatoire et déclenchent la réponse immunitaire appropriée.

1.3.1 Implications pathologiques de l'inflammation

Bien que l'inflammation soit bénéfique pour initier une réponse immunitaire dans un contexte d'infection ou de lésion, elle est souvent impliquée de façon néfaste dans plusieurs processus pathologiques généralement chroniques, comme l'arthrite rhumatoïde, l'athérosclérose, l'ischémie et le cancer à tumeurs solides.

Dans le cas de l'athérosclérose, le cholestérol est initialement emmagasiné dans les macrophages sous-endothéliaux qui se transforment graduellement en cellules spumeuses. La mort de ces cellules entraînera le dépôt de cholestérol à l'intérieur des artères, ce qui déclenchera une réponse immunitaire et la formation, par les cellules musculaires lisses, d'une paroi fibreuse protéique qui recouvre le corps lipidique, qu'on nomme plaque d'athérome. La plaque sera ainsi stable jusqu'à l'intensification de l'inflammation, où des enzymes dégraderont les protéines de la paroi fibreuse et des cytokines entraîneront l'apoptose des cellules musculaires lisses. Des molécules du facteur tissulaire se trouvant dans la plaque seront alors relâchées et le sang coagulera au contact de ces molécules. Le thrombus ainsi

formé risque fortement d'engendrer un accident vasculaire, que se soit un infarctus du myocarde, un accident vasculaire cérébral ou encore une embolie [117].

Lors d'un accident vasculaire, la circulation sanguine à un tissu spécifique est interrompue pour une période indéterminée, soit généralement jusqu'au traitement du patient. Le manque d'oxygène et de nutriments entraîne l'ischémie d'une région plus ou moins grande du tissu affecté. Bien que certains agents thérapeutiques, comme le monoxyde d'azote, entraînent une vasodilatation importante et permettent ainsi une reperfusion partielle temporaire du tissu ischémique, et que la chirurgie par angioplastie amène une reperfusion complète et à long terme du tissu ischémique, les dommages causés par l'interruption de la vascularisation sont fréquents et se manifestent après le rétablissement de la circulation. En effet, l'ischémie favorise l'entrée des cellules en phase nécrotique, ce qui entraîne la relâche de plusieurs enzymes et molécules pro-inflammatoires qui sont normalement à l'intérieur des cellules ou des corps apoptotiques lors d'une mort cellulaire programmée. Alors que les enzymes dégradent la matrice extracellulaire, les molécules pro-inflammatoires relâchées, comme les interleukines et les chimiokines, induisent le recrutement de cellules inflammatoires au site ischémique après la reperfusion. Les leucocytes, particulièrement les neutrophiles, relâcheront alors une multitude d'enzymes et de protéines pour dégrader le milieu et induire une toxicité dans les cellules du tissu ischémique, ce qui entraînera une perte de fonction plus importante du tissu ischémique et une rémission partiellement compromise [118].

Finalement, dans le cas du cancer à tumeur solide, l'inflammation est essentielle pour initier le phénomène d'angiogenèse et elle entraîne ainsi des pronostics plus sévères. En effet, l'inflammation est caractérisée par une vasodilatation des vaisseaux sanguins entourant la tumeur accompagnée par le recrutement de leucocytes. Ainsi, la vasodilatation ainsi que la dégradation du milieu et de la matrice extracellulaire induite par les enzymes des leucocytes activés participent à l'initiation du phénomène d'angiogenèse, ce qui favorise l'expansion tumorale, la relâche de cellules tumorales dans la circulation sanguine et le développement de métastases [5].

1.3.2 Lien entre l'angiogenèse et l'inflammation

Il a été bien établi que l'initiation de l'angiogenèse est dépendante du processus inflammatoire. En effet, la première étape de l'angiogenèse est la vasodilatation et l'augmentation de la perméabilité vasculaire, qui sont deux phénomènes caractéristiques de l'inflammation. De plus, il a été démontré que l'angiogenèse facilite l'inflammation. Ainsi, au contraire de l'angiogenèse physiologique où l'inflammation est présente uniquement pour initier la formation de nouveaux vaisseaux sanguins, l'angiogenèse pathologique est initiée par l'état inflammatoire et participe ensuite à l'intensification de cet état [119]. Ce phénomène contribue ainsi à l'évolution de plusieurs pathologies chroniques comme le cancer tumoral et l'arthrite [114, 120].

1.4 Les neutrophiles

Les neutrophiles sont des leucocytes appartenant à la famille des polymorphonucléaires (PMNs). Ces cellules sont caractérisées par leur noyau polylobé et leurs granules spécifiques qui permettent de différencier les trois types cellulaires qui composent les PMNs, soient les basophiles, les éosinophiles et les neutrophiles. Les basophiles sont caractérisés par des granules qui possèdent un pH basique et qui contiennent une quantité importante d'histamine, une molécule pro-inflammatoire. Ces cellules sont principalement impliquées dans les réactions allergiques et lors de leur dégranulation, l'histamine relâchée entraîne les principaux symptômes allergiques. Pour ce qui est des éosinophiles, leurs granules spécifiques contiennent un pH acide et des médiateurs cytotoxiques. Les éosinophiles participent aux réactions allergiques, ont un rôle protecteur contre certaines infections parasitaires et seraient impliqués directement dans la réponse immunitaire innée et acquise [121].

Les neutrophiles représentent le dernier membre de la famille des PMNs et ils sont de loin les plus abondants; les neutrophiles représentent environ 85% des PMNs, les éosinophiles représentent approximativement 10% des PMNs alors que les basophiles représentent environ 5% de tous les PMNs circulants. Une quantité importante de neutrophiles se retrouvent dans le sang (100 à 700 millions/100 ml) et leur production quotidienne constitutive est de l'ordre de 0,8 à 1,6 x 10^9 neutrophiles/kg de masse corporelle. Cette abondante production permet de

maintenir une concentration plasmatique constante de neutrophiles malgré leur courte demi-vie (10-24 heures dans le sang) [122]. Cependant, cette production peut rapidement augmenter lors de l'initiation d'une réponse immunitaire. En effet, les neutrophiles sont les premières cellules recrutées à un site infecté ou lésé et permettent d'initier une réponse immunitaire rapide et intense. Le processus par lequel les neutrophiles adhèrent et traversent l'endothélium vasculaire pour atteindre le site lésé se nomme diapédèse [123].

1.4.1 Diapédèse des neutrophiles

La diapédèse des leucocytes se produit en trois étapes principales: le roulement des leucocytes, leur adhésion ferme aux CEs et finalement leur transmigration à travers l'endothélium [124]. Les neutrophiles circulants sont tout d'abord dirigés vers les veinules post-capillaires avoisinant le foyer inflammatoire. À ce niveau, il y a un roulement des neutrophiles à la surface des CEs. Le roulement des leucocytes est produit par une famille de molécule d'adhésion, les sélectines. Il existe trois différents types de sélectines, soit la L-sélectine, la P-sélectine et la E-sélectine. La L-sélectine est exprimée constitutivement à la surface des leucocytes et lie deux molécules endothéliales, glyCAM et MAdCAM, qui appartiennent à la super famille des molécules d'adhésion apparentées aux immunoglobulines (super famille Ig). Bien que le roulement des neutrophiles se produise de façon constitutive à l'aide de la L-sélectine, il est fortement augmenté lors d'une réaction inflammatoire suite à la translocation endothéliale de la P-sélectine. En effet, la P-sélectine est

normalement emmagasinée dans les corps de Weibel-Palade, mais suite à un stimulus, elle est transloquée de façon rapide et transitoire à la surface des CEs. La P-sélectine lie alors le PSGL-1 qui se trouve à la surface des leucocytes et qui induit une activation de certaines protéines kinases mitogéniques (p42/44 MAPK) et la relâche d'IL-8. Pour sa part, la E-sélectine est seulement exprimée à la surface des CEs activées suite à divers stimuli inflammatoires tels que les LPS, le TNF-α et l'IL-1β. La E-sélectine lie différents ligands à la surface des leucocytes (PSGL-1 et L-sélectine) et contribue ainsi au roulement des leucocytes en plus de favoriser la transition de l'attachement transitoire à l'endothélium à une adhésion ferme.

Lors du roulement des leucocytes, différentes molécules pro-inflammatoires, telles que l'IL-8 et le PAF, sont relâchées par les CEs et stimulent l'activation des leucocytes. Ces molécules activent aussi directement une catégorie de molécules d'adhésion à la surface des leucocytes, les β_2-intégrines. En effet, lorsqu'activées, ces β_2-intégrines changent de conformation, ce qui augmente leur affinité et leur avidité envers leurs ligands endothéliaux, ICAM-1 et ICAM-2. L'expression de ces deux molécules d'adhésion appartenant à la super famille Ig est différente selon les conditions. En effet, ICAM-1 est constitutivement peu exprimée à la surface des CEs mais plusieurs stimuli pro-inflammatoires, tels que les LPS, le TNF-α et l'IL-1β, stimulent son expression, alors qu'ICAM-2 est exprimée de façon constitutive à la surface des CEs. Ce sont les β_2-intégrines, ICAM-1 et ICAM-2 qui sont responsables de l'adhésion ferme des leucocytes à la surface de l'endothélium et de la transmigration des leucocytes vers le milieu extracellulaire. Finalement, une

autre molécule d'adhésion appartenant à la super famille Ig, la PECAM-1, se retrouve à la surface des CEs et est impliquée dans le processus de diapédèse (transmigration).

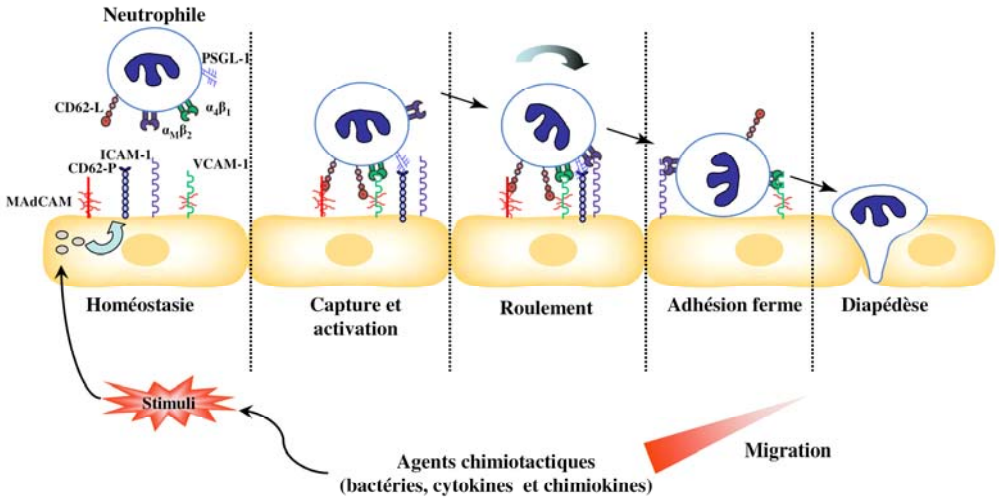

Figure 3. Diapédèse des neutrophiles. Des stimuli proinflammatoires stimulent l'expression endothéliale de la P-sélectine (CD62-P) et de MAdCAM, ce qui permet une adhésion légère des neutrophiles à l'endothélium via le PSGL-1 et la L-sélectine (CD62-L) ainsi que l'initiation du roulement des neutrophiles. Le roulement permet ensuite l'activation et l'adhésion ferme des neutrophiles à l'aide d'intégrines ($\alpha_4\beta_1$ et $\alpha_M\beta_2$) et de molécules appartenant à la super famille Ig (ICAM-1 et VCAM-1). Par la suite, ces molécules d'adhésion permettent la transmigration des neutrophiles et des chimiokines guident les neutrophiles jusqu'au foyer inflammatoire.

1.4.2 Effets pro-inflammatoires des neutrophiles

Une fois au foyer inflammatoire, les neutrophiles activés relâcheront plusieurs chimiokines, telles que l'IL-8 (CXCL8), le GROα (CXCL1), l'IP-10 (CXCL10), le MIG (CXCL9), le MIP-1α (CCL3) et le MIP-1β (CCL4), pour recruter d'autres cellules immunitaires, plus spécifiquement les neutrophiles, les monocytes, les macrophages, les cellules NK, les lymphocytes et les cellules dendritiques immatures [125]. De plus, les neutrophiles relâcheront le contenu de leurs différents granules pour induire une toxicité cellulaire et ainsi détruire les microorganismes. En effet, les neutrophiles possèdent trois différents types de granules, soient les granules azurophiles (primaires), les granules spécifiques (secondaires) et les granules gélatinases (tertiaires) [126]. Les granules azurophiles sont caractérisés par la présence de myéloperoxidase (MPO), qui est une enzyme puissante impliquée dans la respiration oxydative et qui entraîne la formation de radicaux libres cytotoxiques. De plus, les granules azurophiles contiennent la majorité des agents antimicrobiens contenus dans les granules, tels que l'élastase et les α-défensines. Les granules spécifiques sont plutôt caractérisés par la présence importante de lactoferrine, alors que les granules gélatinases contiennent une forte concentration de gélatinase. L'hétérogénéité des granules du neutrophile est dû à leur formation qui s'est produite à différents stades de maturation du neutrophile et la relâche de leur contenu n'est pas contrôlée par les mêmes stimuli [127]. Bien que plusieurs enzymes les différencient, les trois granules contiennent du lysozyme et ils ont tous un pouvoir cytotoxique puissant. En plus des granules, les neutrophiles possèdent aussi des vésicules sécrétoires qui contiennent plusieurs

protéines membranaires et des protéines plasmatiques. Ces vésicules permettent aux neutrophiles d'exprimer plusieurs molécules nécessaires pour leur diapédèse et leur activation telles que la β_2-intégrine et des récepteurs de chimiokines [127].

Bien que la dégranulation et la respiration oxydative soient essentielles à la résolution d'une infection, elle engendre une toxicité cellulaire au site d'infection ou de blessure et des dommages importants et même irréversibles peuvent apparaître lors d'une inflammation chronique [128]. Cette inflammation chronique est d'ailleurs la cause principale de plusieurs maladies, notamment l'arthrite rhumatoïde, la maladie de Crohn et le syndrome de réponse inflammatoire systémique (SIRS). Pour éviter de causer des dommages indésirables, les neutrophiles ont une durée de vie très courte et entrent dans un processus de mort programmée (processus apoptotique) pour éviter de déverser leur contenu catalytique dans le milieu extracellulaire. En effet, la demi-vie des neutrophiles circulants est d'environ 8 à 20 heures, alors que lorsqu'ils sont activés, les neutrophiles tissulaires ont une durée de vie de 1 à 4 jours [129]. Cependant, l'apoptose des neutrophiles est un processus finement régulé et plusieurs molécules biologiques peuvent prévenir l'apoptose et ainsi augmenter la viabilité des neutrophiles et leur pouvoir cytotoxique. De plus, de récentes études ont démontré que l'entrée des neutrophiles dans la phase apoptotique induisait la relâche de molécules anti-inflammatoires, telles que les lipoxines, ce qui a pour effet de favoriser la résorption du foyer inflammatoire [130]. La longévité des neutrophiles est ainsi devenue une cible thérapeutique de choix pour plusieurs maladies

inflammatoires et plusieurs groupes de recherche s'intéressent aux mécanismes et aux médiateurs de l'apoptose spontanée des neutrophiles [131-134].

1.4.3 Médiateurs de l'apoptose des neutrophiles

Les neutrophiles en apoptose spontanée sont caractérisés par plusieurs phénomènes de l'apoptose classique. En effet, lors de l'entrée en phase apoptotique, les neutrophiles présentent une diminution de leur taille, une externalisation des phosphatidylsérines des membranes plasmiques, une condensation du noyau, une dépolarisation des mitochondries, une vacuolisation du cytoplasme et une fragmentation de l'ADN [135, 136]. De plus, les neutrophiles apoptotiques deviennent peu fonctionnels, c'est-à-dire que leurs capacités de migration, de dégranulation et de respiration oxydative sont grandement diminuées [137, 138]. Plusieurs molécules intracellulaires sont associées à l'apoptose des neutrophiles, soient les espèces réactives de l'oxygène (ROS), les caspases, les protéines de la famille Bcl-2, les calpaïnes, la voie PI3K/Akt, la voie p38 MAPK et la protéine kinase C (PKC-δ) [139].

En effet, une concentration élevée de ROS présente dans les neutrophiles est directement liée à l'induction de l'apoptose. De ce fait, un traitement pharmacologique avec des inhibiteurs de ROS, tels que la catalase et le glutathione, retarde significativement l'entrée en apoptose des neutrophiles [140-142]. De plus, les individus présentant un débalancement en faveur des oxydants par rapport aux

antioxydants, comme par exemple les personnes âgées, ont un taux d'apoptose de neutrophiles plus élevé que la moyenne [143]. Bien que les mécanismes exacts de l'induction de l'apoptose par les ROS ne soient pas clairement définis, l'altération de l'ADN induite par les ROS pourrait être responsable de l'induction de l'apoptose en activant la molécule p53, qui est un inducteur classique de l'apoptose lors de dommages génotoxiques [144, 145]. Une autre hypothèse serait que les ROS activent directement certains mécanismes intracellulaires impliqués dans la survie et l'apoptose cellulaire, soient NF-κB et ERK [146-148]. Finalement, il a été démontré que dans les neutrophiles, les ROS induisent la synthèse de céramides, ce qui amène les récepteurs de mort à se rassembler (*clustering*) et à activer la caspase-8, permettant ainsi le déclenchement de la cascade des caspases [149, 150]. Les caspases activées, particulièrement la caspase-3, induisent alors la condensation de la chromatine et la fragmentation de l'ADN, ce qui entraînent l'initiation du processus apoptotique [151]. Cependant, il a aussi été suggéré que les ROS pourraient induire l'apoptose des neutrophiles par des mécanismes indépendants des caspases puisque différents groupes de recherche ont démontré qu'une forte concentration de ROS ou d'espèces réactives de l'azote (RNS) inhibe l'activité des caspases [152, 153].

Les caspases sont des protéines clés de l'apoptose dans une grande majorité de types cellulaires. L'activation de la cascade des caspases se produit de deux façons distinctes, soient la voie extrinsèque des récepteurs de mort et la voie intrinsèque apoptotique associée aux mitochondries [139]. La voie extrinsèque est

principalement produite par les récepteurs du TNF (TNFR) et Fas qui activent la caspase-8 et déclenche ainsi la cascade des caspases. Pour sa part, la voie intrinsèque est caractérisée par une perte du potentiel membranaire mitochondrial, ce qui entraîne la relâche d'un facteur pro-apoptotique puissant dans le cytosol, le cytochrome c. Par la suite, le cytochrome c déclenche la cascade des caspases et il a été suggéré que, dans les neutrophiles, l'activation de la caspase-9 soit responsable de l'initiation de cette cascade [154]. Il a été démontré que l'apoptose spontanée des neutrophiles est médiée par la voie intrinsèque plutôt que par la voie extrinsèque [154, 155]. Finalement, contrairement à l'apoptose induite par le TNF-α où les caspases sont essentielles, une controverse existe toujours à savoir si l'implication des caspases est essentielle dans l'apoptose spontanée [139, 156].

Les protéines de la famille Bcl-2 sont des molécules clés dans la survie cellulaire et sont impliquées dans la voie intrinsèque apoptotique où elles peuvent induire une dysfonction mitochondriale. En effet, la famille Bcl-2 est séparée en deux catégories distinctes; les protéines pro-apoptotiques, telles que Bid et Bax, et les protéines anti-apoptotiques, telles que Bcl-2, Mcl-1, A1 et Bcl-χL. Bien que les neutrophiles n'expriment pas Bcl-2, ils expriment une quantité importante de Mcl-1 et cette quantité diminue lors de l'entrée en apoptose des neutrophiles [157]. En effet, à l'état viable, Mcl-1 forme un complexe hétérodimérique avec la protéine Bax, mais lorsque les niveaux de Mcl-1 diminuent, Bax est relâchée et transloquée à la membrane mitochondriale où elle induit une perméabilisation de la membrane mitochondriale externe et la relâche subséquente du cytochrome c [158-160]. Le

cytochrome c formera alors un complexe nommé apoptosome qui activera la caspase-9 et déclenchera ainsi la cascade des caspases [161, 162]. De plus, plusieurs facteurs anti-apoptotiques, tels que NF-κB et PI3K, ont la capacité d'induire l'expression et la synthèse de Mcl-1 [163].

Les calpaïnes sont des enzymes exprimées constitutivement par les neutrophiles mais sont peu actives dans les neutrophiles viables en raison du niveau élevé de calpastatine, un inhibiteur spécifique des calpaïnes [164, 165]. Cependant, lors de l'apoptose des neutrophiles, les niveaux de calpastatine diminuent significativement et les calpaïnes activées favorisent ainsi l'apoptose [164]. En effet, les calpaïnes libèrent le fragment actif de Bax, en plus d'empêcher la liaison subséquente de Bax avec la protéine anti-apoptotique Bcl-χL [166]. L'activation de Bax entraîne ensuite la relâche du cytochrome c, ce qui induira l'activation de la caspase-3 [166]. De plus, les calpaïnes désactivent le *x-linked inhibitor of apoptosis* (XIAP), qui est une molécule qui se lie aux caspase-3, 7 et -9 et qui bloque leur activité [167].

1.4.4 Voies de signalisation de l'apoptose des neutrophiles

Une des principales voies de signalisation impliquées dans l'apoptose spontanée des neutrophiles est la voie PI3K/Akt. En effet, il a été démontré que l'activité du facteur anti-apoptotique protéine kinase B (PKB)/Akt diminue fortement lors de l'apoptose des neutrophiles [139]. De plus, un traitement pharmacologique avec des inhibiteurs de PI3K induit l'apoptose des neutrophiles [168]. On peut aussi

observer que des souris déficientes en PI3Kγ, qui sont caractérisées par une diminution de l'activité d'Akt, présentent une augmentation du taux d'apoptose de leurs neutrophiles [154, 169]. D'ailleurs, plusieurs agents anti-apoptotiques, tels que les LPS, le GM-CSF et le G-CSF, retardent l'apoptose des neutrophiles en ramenant l'activité d'Akt et/ou d'ERK à un niveau plus élevé [170-173]. Une fois Akt activée, elle induit la phosphorylation d'une multitude de protéines sous-jacentes, dont certaines impliquées dans les voies de survie et de mort cellulaire, comme par exemple Bax, Forkhead and NK-κB [174, 175]. La phosphorylation de Bax favorise ainsi son hétérodimérisation avec Mcl-1, Bcl-χ1 et A1, ce qui inhibe son activité [157] (Figure 4).

La voie p38 MAPK est aussi impliquée dans l'apoptose spontanée constitutive des neutrophiles et a un rôle anti-apoptotique, bien que ce rôle soit controversé [176, 177]. En effet, bien que des agents de stress cellulaires, tels que l'hyperosmolarité et l'irradiation, accélèrent l'apoptose en activant fortement la voie p38 MAPK [178], des inhibiteurs de cette voie favorisent également l'entrée des neutrophiles en apoptose spontanée [179]. De plus, il a été suggéré que la voie p38 MAPK favorise la survie des neutrophiles en induisant la phosphorylation de la caspase-3 et de la caspase-8, ce qui inhibe leur activité [180]. Finalement, une isoforme de la protéine kinase C (PKC), la PKC-δ, est aussi un médiateur essentiel de l'apoptose des neutrophiles [154]. En effet, cette protéine est activée par la caspase-3 au cours de l'apoptose spontanée [181]. De plus, des inhibiteurs spécifiques de la PKC inhibent l'apoptose spontanée des neutrophiles [182].

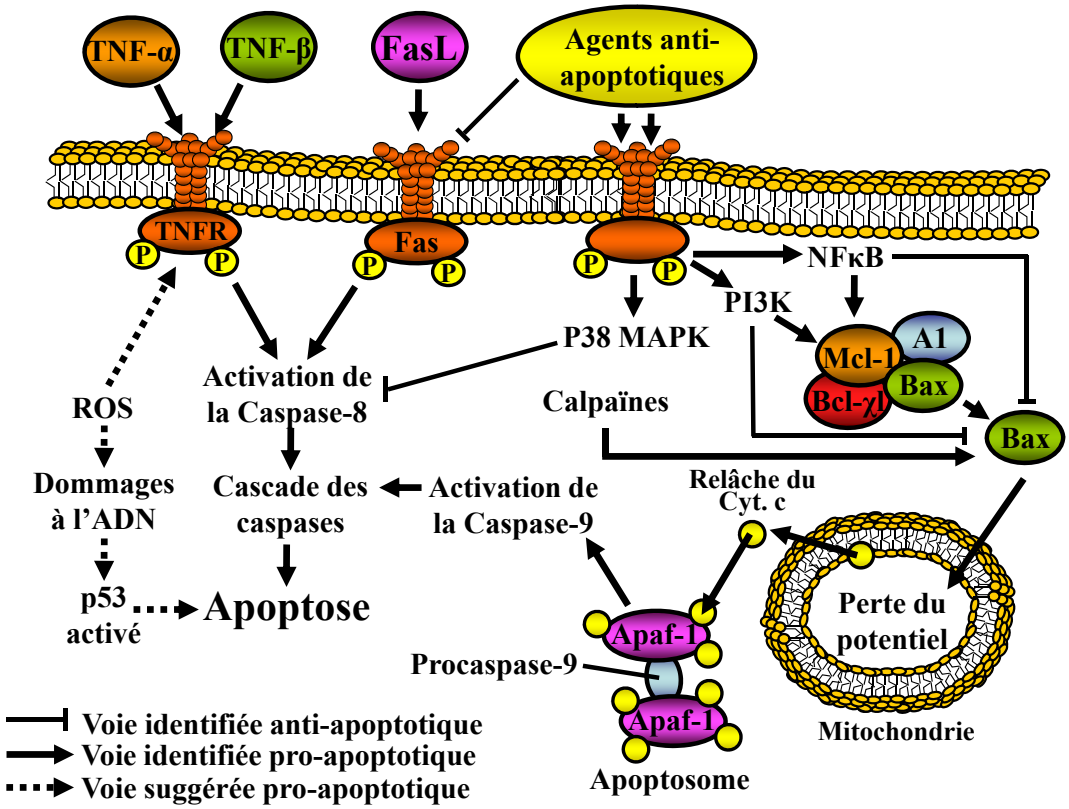

Figure 4. Voies et mécanismes de l'apoptose spontanée des neutrophiles. Les ROS induisent l'apoptose via l'activation de p53 suite à des dommages au niveau de l'ADN [144, 145] ou via l'activation des récepteurs de mort (TNFR, Fas et TRAIL) [149, 150]. Les caspases déclenchent l'apoptose par deux mécanismes distincts; la voie extrinsèque, c'est-à-dire l'activation de récepteurs de mort et la voie intrinsèque qui est associée à une perte du potentiel membranaire mitochondrial [139]. De plus, un membre de la famille Bcl-2, soit la protéine pro-apoptotique Bax, peut être relachée du complexe qu'elle forme avec Mcl-1, A1 et Bcl-χl et induire la perte du potentiel membranaire mitochondrial et déclencher l'apoptose [157]. Les voies PI3K et NF-κB stimulent d'ailleurs l'expression de la protéine anti-apoptotique Mcl-1 qui inhibe l'activité de Bax [163]. Finalement, les calpaïnes induisent l'apoptose en favorisant la relâche de Bax [166].

1.4.5 Modulation de la survie des neutrophiles par des stimuli extracellulaires

Bien que la viabilité des neutrophiles circulants soit très courte, les neutrophiles présents dans un foyer inflammatoire ont une durée de vie significativement plus longue. En effet, plusieurs molécules pro-inflammatoires et facteurs de croissance augmentent la durée de vie des neutrophiles, ce qui a pour effet de prolonger l'inflammation. Les interférons de type 1 et 2 retardent l'apoptose des neutrophiles en activant STAT3 et en favorisant l'expression du *cellular inhibitor of apoptosis 2* (cIAP2) [183]. De plus, plusieurs agonistes des TLR, tels que les LPS, les peptidoglycans, le R-848 et le CpG-DNA, retardent aussi l'apoptose des neutrophiles. L'activation des TLR retarde l'apoptose des neutrophiles par plusieurs mécanismes, soient l'induction de la phosphorylation d'Akt et une augmentation des médiateurs anti-apoptotiques Mcl-1 et A1 [184, 185]. Plusieurs interleukines retardent également l'apoptose spontanée des neutrophiles, soit l'IL-1β, l'IL-2, l'IL-6, l'IL-8 et l'IL-15 [186-190]. Dans le cas de l'IL-8, la prolongation de la viabilité des neutrophiles est médiée en inhibant l'activité pro-apoptotique des interactions de Fas et FasL et des récepteurs du TNF-α [191]. De plus, des lipides bioactifs, tels que le PAF et le LTB_4, augmentent également la viabilité des neutrophiles [192, 193]. En effet, l'effet de survie induit par le PAF serait dépendant de la voie Akt puisque des inhibiteurs d'ERK et d'Akt inhibent l'effet du PAF [192]. Différents facteurs de croissance, comme par exemple le GM-CSF et le G-CSF, peuvent aussi retarder l'apoptose spontanée des neutrophiles [187]. En effet, le GM-CSF favorise l'expression de la voie PI3K/Akt et inhibe l'expression de plusieurs facteurs pro-apoptotiques [171, 172], alors que le G-CSF

prolongerait la survie des neutrophiles en inhibant les caspases et en bloquant la redistribution de Bid/Bax [160]. Finalement, plusieurs autres molécules et activités peuvent retarder l'apoptose spontanée des neutrophiles, comme la leptine, des niveaux extracellulaires élevés de potassium, l'adhésion des neutrophiles et l'hypoxie (Table I) [194-197].

Plusieurs stimuli peuvent également accélérer l'apoptose spontanée des neutrophiles. L'apoptose induite des neutrophiles est généralement induite par des récepteurs de mort membranaires, plus précisément le récepteur Fas et son ligand FasL. En effet, les neutrophiles expriment constitutivement de hauts niveaux de ces deux molécules et la stimulation du FasL induit une apoptose rapide des neutrophiles [198, 199]. L'induction de l'apoptose par FasL serait due à l'activation de la caspase-3 et de la caspase-8 ainsi qu'à une augmentation de la perméabilité mitochondriale, qui serait induite par une activation de la protéine Bid [200, 201]. D'autres récepteurs de mort membranaire peuvent aussi être impliqués dans l'induction de l'apoptose des neutrophiles. En effet, lorsque les récepteurs TRAIL-R2 et TRAIL-R3 lient leur ligand TRAIL (*TNF-related apoptosis-inducing ligand*), on observe une augmentation de l'apoptose qui serait causée par l'inhibition des effets anti-apoptotiques de différentes cytokines [202-206]. La phagocytose par les neutrophiles de différents pathogènes, tels que l'*Escherichia coli,* les *Staphylococcus aureus*, les *Mycobacterium tuberculosis* et les virus influenza, entraîne aussi une accélération de l'apoptose des neutrophiles en

augmentant la production de ROS et en induisant l'activation de la caspase-3 et de la caspase-8 (Table I) [140, 146, 207-209].

Finalement, le TNF-α exerce un effet unique sur la viabilité des neutrophiles. En effet, selon les conditions, le TNF- α peut soit favoriser la survie ou l'apoptose des neutrophiles [210]. Ainsi, en présence de faibles concentrations de TNF- α, on observe généralement une augmentation de la viabilité des neutrophiles induite par une augmentation de l'activité PI3K, une activation de PKC-δ et d'ERK1/2, une relâche d'IL-8 ainsi que par une augmentation de l'expression des protéines anti-apoptotiques A1 et Bcl-χl [211-215]. À l'opposé, à plus fortes concentrations, le TNF- α induit plutôt l'apoptose des neutrophiles en favorisant la synthèse de ROS et en activant JNK MAPK (Table I) [216-218].

Agents anti-apoptotiques	**Mécanismes Impliqués**
Interférons (type 1 et 2)	↑ STAT3 ↑ expression de cIAP2
Agonistes des TLR (LPS, CpG DNA)	↑ Akt ↑ Mcl-1 et A1
GM-CSF	↑ PI3K/Akt
G-CSF	↓ activité des caspases
IL-8	↓ activité Fas/FasL ↓ activité TNFR
PAF	↑ Akt ↑ ERK
TNF-α	↑ PI3K ↑ A1 et Bcl-χl ↑ IL-8

Agents pro-apoptotiques	**Mécanismes impliqués**
Fas/FasL	↑ activité des caspases 3 et 8 ↑ Bid
TRAILR 2/3 et TRAIL	↓ effets anti-apopt. de cytokines
Pathogènes	↑ ROS ↑ activité des caspases 3 et 8
TNF-α	↑ ROS ↑ JNK MAPK

Table I. Médiateurs et mécanismes anti et pro-apoptotiques

Bien que l'effet de différents agents pro et anti-apoptotiques soit bien identifié sur les neutrophiles *in vitro*, un environnement *in vivo* comprend généralement une multitude d'agents aux effets opposés et aux mécanismes différents. En effet, dans une telle situation, la balance des facteurs pro et anti-apoptotiques décidera du sort des neutrophiles. Mentionnons que dans le cas d'un foyer inflammatoire, la balance est généralement en faveur des agents anti-apoptotiques et la présence

d'hypoxie est un facteur prédominant qui induit une forte diminution de l'apoptose des neutrophiles [219]. La prolongation de la survie des neutrophiles facilitera une prolongation et une intensification de l'état inflammatoire, ce qui pourrait mener à des dommages tissulaires importants, que se soit dans un cas d'inflammation aiguë, comme par exemple dans les pathologies du SIRS et du choc septique [220-223], ou encore dans un contexte d'inflammation chronique, tels que dans l'arthrite rhumatoïde et la maladie de Crohn [224-226]. Ainsi, une nouvelle approche thérapeutique se développe présentement pour traiter certaines maladies inflammatoires avec des inhibiteurs de l'apoptose des neutrophiles, comme par exemple l'utilisation d'inhibiteurs des CDK (*cyclin-dependent kinases*) (CDKi) dans des modèles murins inflammatoires [227]. En effet, il a été démontré que les CDKi accélèrent l'entrée des neutrophiles en apoptose et que leurs utilisations dans des modèles murins d'arthrite, d'inflammation pulmonaire et de pleurésie aiguë favorisent significativement la résolution de l'inflammation [227]. L'apoptose des neutrophiles est donc une cible de choix comme traitement pour les maladies inflammatoires et il reste encore beaucoup à découvrir sur la régulation et les mécanismes de l'apoptose spontanée des neutrophiles.

1.5 But du travail de recherche

Il a été démontré qu'Ang1 et Ang2 avaient la capacité de moduler la survie des CEs. En effet, Ang1 prolonge la viabilité des CEs [103, 104], alors que l'effet d'Ang2 sur la survie des CEs est dépendant des conditions expérimentales et peut ainsi retarder ou encore accélérer la mort des CEs [96, 98, 100, 101, 105]. Nous avons récemment démontré l'expression du récepteur des angiopoïétines, le récepteur Tie2, à la surface des neutrophiles [107]. Nous avons également démontré qu'Ang1 et Ang2 induisent la synthèse du PAF dans les CEs et les neutrophiles [106, 107]. Sachant que les angiopoïétines ont la capacité de moduler la viabilité de certains types cellulaires et que le PAF est un agent anti-apoptotique reconnu pour les neutrophiles [192], le but de mon travail de recherche consistait donc à déterminer l'effet d'Ang1 et d'Ang2 sur la viabilité des neutrophiles et d'identifier les médiateurs de cet effet, alors que mon hypothèse suggérait que les angiopoïétines prolongeraient la viabilité des neutrophiles. De plus, nous avons démontré que les angiopoïétines induisent la migration des neutrophiles et potentialisent l'effet de l'IL-8 sur la migration des neutrophiles [112]. Ainsi, puisque l'IL-8 est un agent anti-apoptotique connu [186], nous avons également voulu déterminer si la combinaison d'IL-8 et des angiopoïétines peut avoir un effet synergique ou additif sur la viabilité des neutrophiles et déterminer si cette cytokine participe à l'effet potentiel d'Ang1 et d'Ang2 sur la survie des neutrophiles.

2.0 ARTICLE

Angiopoietin-1 but not angiopoietin-2 promotes neutrophil viability: Role of interleukin-8 and platelet-activating factor

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ABSTRACT

We previously reported the expression of angiopoietin receptor Tie2 on human neutrophils. Both angiopoietins (Ang1 and Ang2) induce platelet activating factor (PAF) synthesis from endothelial cells (ECs) and neutrophils. Both angiopoietins can also modulate ECs viability and since PAF can promote pro-survival activity on neutrophils, we addressed whether Ang1 and/or Ang2 could modulate neutrophil viability. Neutrophils were isolated from venous blood of healthy volunteers and neutrophil viability was assessed by flow cytometry using apoptotic and necrotic markers (annexin-V and propidium iodide (P.I.), respectively). Basal neutrophil viability from 0 to 24 hours post-isolation decreased from 98% to ≈45%. Treatment with anti-apoptotic mediators such as interleukin-8 (IL-8; 25 nM) and PAF (100 nM) increased neutrophil basal viability by 34 and 26% (raising it from 43 to 58 and 55%) respectively. Treatment with Ang1 (0.001 – 50 nM) increased neutrophil viability by up to 41%, while Ang2 had no significant effect. Combination of IL-8 (25 nM) or PAF (100 nM) with Ang1 (10 nM) further increased neutrophil viability by 56 and 60% respectively. We also observed that Ang1, but not Ang2 can promote IL-8 release and that a pretreatment of the neutrophils with blocking anti-IL-8 antibodies inhibited the anti-apoptotic effect of IL-8 and Ang1 by 92 and 81% respectively. Pretreatment with a selective PAF receptor antagonist (BN 52021), did abrogate PAF pro-survival activity, without affecting Ang1-induced neutrophil viability. Our data are the first one to report Ang1 pro-survival activity on neutrophils, which is mainly driven through IL-8 release.

Key words: **angiopoietins, interleukin-8, neutrophil, viability, apoptosis, inflammation.**

1. INTRODUCTION

Angiopoietins (Ang1 and Ang2) are growth factors structurally related, and contributing to angiogenesis upon binding to Tie2 receptor [1]. Ang1 is known as a Tie2 agonist capable of inducing EC migration and proliferation [2-5]. In addition, Ang1 promotes pericytes recruitment to the newly formed vessels, therefore leading to the maturation and stabilization of the vasculature [6, 7]. Furthermore, Ang1 has the capacity to reduce apoptosis of many cellular types such as EC, cardiomyocytes and mesenchymal stem cells [8-11]. This activity can lead to a protective role of Ang1 in acute inflammatory conditions such as in lung injury, and in chronic diseases such as cardiac remodeling where Ang1 improves cardiomyocytes survival and reduces cardiac hypertrophy [12, 13]. In contrast, Ang2 was initially reported as a Tie2 antagonist, inducing vascular permeability and EC apoptosis, therefore, leading to the promotion of a pro-inflammatory environment and the destabilization of pre-existing vasculature [14, 15]. However, mounting evidences demonstrate that Ang2 could also have agonistic properties by being able to induce Tie2 phosphorylation, EC chemotaxis and *in vitro* tubule capillary-like formation [16-19]. In previous studies, we reported that both angiopoietins exert pro-inflammatory activities on ECs by their capacity to stimulate PAF synthesis and P-selectin translocation, therefore leading to neutrophil adhesion onto activated ECs and transmigration [20-22]. Furthermore, angiopoietins can also modulate cytokine activities in ECs, for instance, at low concentrations, Ang2 is a priming factor for

tumor necrosis factor-α (TNF-α) [23] and both Ang1 and Ang2 have been reported to potentiate VEGF-induced angiogenesis in a mouse cornea model [1].

We also reported the expression of Tie2 receptor on human neutrophil, and that both angiopoietins can promote pro-inflammatory activities on neutrophils [22]. Ang1 and Ang2 support neutrophil PAF synthesis, activation of CD11b/CD18 (β_2-integrin), adhesion and migration [22, 24]. In addition, angiopoietins can also enhance the activities of pro-inflammatory mediators, for instance, pretreatment with Ang1 and/or Ang2 potentiates IL-8-induced neutrophil migration [24].

Neutrophil lifespan is relatively short, ranging from 8 to 20 hours in blood circulation and up to few days in perivascular tissues [25-27]. Neutrophils are the primary leukocytes being recruited at inflammatory sites and promote a range of pro-inflammatory activities, namely by their capacity to release various chemokines and cytokines, including VEGF, TNF-α, IL-1, IL-8 and PAF [28, 29]. Neutrophils can also secrete catalytic enzymes (metalloproteinases, lysosymes), which contribute to extracellular matrix degradation and elimination of pathogens [29, 30]. Increasing neutrophil viability can prolong and enhance the inflammatory response, thus, contributing to the development and progression of multiple vascular-associated diseases such as systemic inflammatory response syndrome (SIRS), tumor growth, ischemia/reperfusion injury and rheumatoid arthritis (RA) [26, 31-35].

For instance, it has been reported that the expression of both angiopoietins and members of Tie-receptor family (Tie1 and Tie2) is increased in patients

suffering from RA, and that the corresponding expression is not limited to vascular cells, but also overexpressed in stromal cells and in the synovial lining [36]. Such overexpression could lead to the development of pathological angiogenesis, which can be inhibited by the blockade of Ang/Tie ligand-receptor system [37]. In addition, since RA is characterized as a chronic inflammatory disease, angiopoietins could contribute to the inflammatory process through their capacity to promote neutrophil-mediated pro-inflammatory responses, namely through PAF and IL-8 participation [22, 24, 38, 39].

PAF is a bioactive lipid that derivates from arachidonic acid metabolism acting through the stimulation of a G-protein-coupled receptor (PAFR). This potent pro-inflammatory mediator is involved in inflammation, reproduction, angiogenesis and tumor growth metastasis [40]. PAF activates and is produced by many inflammatory cells including ECs, platelets, monocytes, lymphocytes and neutrophils. In addition, in function of the cell type, PAF promotes many pro-inflammatory activities such as platelet aggregation and modulation of cell viability [41]. For instance, PAF promotes apoptosis on hepatocytes and neurons, whereas it can prolong cell viability of various leukocytes, including neutrophils [40, 42].

IL-8 was first described as a monocyte-derived neutrophil chemotactic factor (MDNCF) [43-45], and subsequently reported for being synthesized by ECs, fibroblasts and keratinocytes [46, 47]. IL-8 is a major pro-inflammatory mediator, acting predominantly on neutrophils where it induces respiratory burst [48], generation of superoxide and hydrogen peroxide [49], cellular reorganization [49], secretion of catalytic enzymes (metalloproteinases, lysosymes) [50], synthesis of

bioactive lipids [51], translocation and activation of adhesion molecules [52], chemotaxis [53] and pro-survival activity [54]. IL-8 is also involved in the development of various pathological conditions, such as acute respiratory distress syndrome (ARDS), ischemia/reperfusion injury, tumor growth and metastasis [46, 54].

Since both angiopoietins can modulate endothelial cell survival [10, 11, 17, 55-57] and induce PAF synthesis [21, 22] which supports neutrophil viability [42], and that both angiopoietins can modulate IL-8 chemotactic activity on neutrophils [24], we thus wanted to assess whether Ang1 and/or Ang2 can modulate neutrophil viability, and if so, delineate the potential contribution of specific cytokines being synthesized and released by the neutrophils.

2. MATERIAL & METHODS

2.1 Neutrophil isolation and purification

Human venous blood was obtained from consent healthy donors free from medication for at least 10 days prior to the experiments and in accordance with the guidelines of the Montreal Heart Institute's ethical committee. Neutrophils were isolated as described previously [22, 24, 58]. Neutrophils were then resuspended in RPMI medium (Lonza, Basel, Switzerland) supplemented with 25 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and 1% penicillin/streptomycin. At least 95% of isolated cells were neutrophils as determined by staining with Kwik Diff solutions according to manufacturer's instructions (Thermo Fisher Scientific, Kalamazoo, MI, USA) and viability was found to be greater than 98% as assessed by Trypan blue dye exclusion assay [59].

2.2 Assessment of viability, apoptosis and necrosis

Neutrophils were cultured in 24-well plates (Becton Dickinson, San Jose, CA, USA) at 5 x 10^6 cells/well for different periods of time (0-24 hours) at 37°C and treated with various agonists: Ang1 and Ang2 (R&D Systems, Minneapolis, MN, USA), IL-8 (Peprotech, Rocky Hill, NJ, USA) and methylcarbamyl-PAF C-16 (PAF; Cayman Chemical, Ann Arbor, MI, USA) in RPMI medium supplemented with 10% human serum (Millipore, Billerica, MA, USA). In some experiments, neutrophils were preincubated with a secreted phospholipase A_2 ($sPLA_2$) inhibitor, 12-epi-scalaradial (Biomol, Plymouth Meeting, PA, USA), a cytosolic PLA_2

($cPLA_2$) inhibitor, $AACOCF_3$ (Cayman Chemical), a PAF receptor antagonist, BN 52021 (Sigma-Aldrich, St. Louis, MO, USA), a goat polyclonal blocking anti-human Tie2 antibody (R&D Systems, #AF313), a goat polyclonal blocking anti-human IL-8 antibody (R&D Systems, #AB-208-NA) or control IgG isotype for 30 minutes to 2 hours prior to the addition of selected agonists. Viability was measured by flow cytometry using annexin-V-fluorescein isothiocyanate (FITC) and P.I. (Becton Dickinson) as previously described [60, 61]. Briefly, neutrophils (1×10^6 cells/50 µl) were washed with PBS, centrifuged (900*g*, 7 minutes at 4°C), resuspended in annexin-V binding buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) (Becton Dickinson) and incubated in the dark for 15 minutes at room temperature in the presence of 2.5 µl of annexin-V-FITC (20 µg/ml in aqueous buffered solution containing BSA and 0.09% sodium azide) and 5 µl of P.I. (50 µg/ml in PBS, pH 7.4) (PharMingen, Becton Dickinson). Flow cytometry analyses (15 000 events per tube) were performed using a flow cytometer with a 488 nm wavelength argon laser (Coulter EPICS XL, Beckman Coulter, Brea, CA, USA). Intact neutrophils were selected according to size and granularity. Cells negative for both annexin-V and P.I. were considered as viable (annexin-V^-/P.I.$^-$), annexin-V-positive and P.I.-negative cells were considered as early apoptotic (annexin-V^+/P.I.$^-$) and cells positive for both markers were considered as necrotic and/or in late apoptotic phase (annexin-V^+/P.I.$^+$).

2.3 Morphological assessment of neutrophil apoptosis

Viability was also assessed by morphological observations as previously described [27]. Neutrophils (5 x 10^5 cells/condition) were aspirated, cytocentrifuged, fixed and stained for 30 seconds with hematoxylin QS (Vector Laboratories, Burlingame, CA, USA). Neutrophils were examined under oil immersion light microscopy at 400X magnification. Cells in five random fields per condition were analyzed and apoptotic/necrotic cells were characterized by condensed and darkly stained nuclei. At least 400 neutrophils were counted per condition. Flow cytometry and morphological assessment gave similar results in regard to neutrophil viability.

2.4 Neutrophil chemotactic activity

In vitro chemotactic assays were performed in Transwell-96 well permeable supports. The top and the bottom wells were separated by a porous polycarbonate membrane filter (3 μm pore size) (Corning Life Science, Corning, NY) as previously described [62, 63]. Briefly, the bottom wells were loaded with RPMI containing PBS, IL-8 (25 nM) or fMLP (0.1 – 10 μM) to a final volume of 230 μl. The top wells were loaded with neutrophils (10^7 cells/ml; 80 μl from a RPMI suspension). In function of the experiments, neutrophils were freshly added post-isolation or pretreated for 24 hours with PBS or Ang1 (10 nM) prior to the chemotactic assay. The Transwell-96 well permeable supports were incubated at 37°C for 1 hour in a humidified incubator in the presence of 5% CO_2. At the end of

the incubation period, the supernatant from the bottom wells was collected, and a count of migrating neutrophils was assessed with an Amnis ImageStream system.

2.5 Statistical analysis

Results from each experiment are the mean of cumulative independent experiments from the blood of at least three independent donors, and expressed as the mean ± SEM. Comparisons were made by analysis of variance (ANOVA) followed by a Dunnett's test. Differences were considered significant at p values ≤ 0.05.

3. RESULTS

3.1 Ang1, but not Ang2, promotes neutrophil viability

It has been reported that Ang1 and Ang2 modulate ECs survival while PAF increases neutrophil viability [10, 11, 17, 42, 56, 57, 64]. On our side, we have reported that both angiopoietins induce PAF synthesis in neutrophils [22]. Thus, we sought to determine whether angiopoietins could modulate neutrophil viability and the potential role of PAF. In a first series of experiments we assessed the survival rate of neutrophils on a 24-hour period. Upon neutrophil isolation, the viability of the cells was ≈98% and declined time-dependently to ≈45% after 24 hours. We then used PAF as a positive control to promote neutrophil viability. Upon a 24-hour incubation period, we observed a bell-shape concentration-dependent anti-apoptotic effect of PAF (1 – 10 000 nM) on neutrophil viability with an optimal effect being achieved at 100 nM, providing a 26% increase of neutrophil viability (raising the basal viability from 43.4 to 54.8%) (**suppl. data Fig. 1**).

We then assessed the capacity of both angiopoietins (Ang1 and Ang2) to modulate neutrophil viability on a concentration- and time-dependent manner. Treatment with Ang1 (0.001 – 50 nM; 0.07 – 3500 ng/ml) increased significantly neutrophil viability at 1, 10 and 50 nM, raising the basal viability from 43.4 to 53.3, 59.0 and 61.3% respectively (corresponding to a 23, 36 and 41% increase). Interestingly, in the current set of experiments, Ang1 at 10 nM was already more potent than PAF (100 nM) to promote neutrophil viability (**Fig. 1A**). To assess whether Ang1-mediated pro-survival activity was caused by an effect on apoptosis

or necrosis, we evaluated the effect of Ang1 on neutrophil viability with two specific markers of membrane asymmetry, annexin-V-FITC (phosphatidylserine externalization; apoptosis) and P.I. (intercalating agent; necrosis). These data were further confirmed by cytologic staining and morphological assessment of neutrophil viability under optical microscopy. The beneficial effect of Ang1 on neutrophil viability is observed by its capacity to reduce the apoptotic and necrotic events. In fact, PAF and Ang1 had a similar capacity to significantly lower neutrophil apoptosis (reducing it from 38.9% to 34.1% and 29.4% respectively). In addition, PAF and Ang1 had a similar capacity to significantly lower neutrophil necrosis (reducing it from 15.9% to 8.8% and 9.7% respectively) (**Fig. 1A**). In parallel, we performed the same experiments with neutrophils from the same donors and observed that Ang2 (0.001 – 50 nM; 0.066 – 3300 ng/ml) as opposed to Ang1, had no significant effect on neutrophil viability (**Fig. 1B**). As the maximal effect mediated by Ang1 was nearly achieved at 10 nM, we elected to limit the use of Ang1 up to 10 nM for upcoming experiments.

We then treated the neutrophils with the angiopoietins (Ang1, Ang2; 1 and 10 nM) and PAF (100 nM; positive control) on a 24-hour time period to address the kinetic of Ang1-mediated anti-apoptotic activity. Treatment with Ang1 (1 and 10 nM; 70 and 700 ng/ml) provided a positive trend on neutrophil viability upon 12 hours post-isolation, and the Ang1 anti-apoptotic effect was significant and maximal (10 nM) at 24 hours **(Fig. 2A)**. By performing a time-response kinetic, we were led to observe that Ang1 (1 and 10 nM) had a beneficial pro-survival activity by delaying the entry of neutrophils into apoptotic and necrotic phase **(Fig. 2B-C)**.

Treatment with PAF (100 nM) or Ang1 (1 and 10 nM) at 24 hours significantly reduced neutrophil apoptosis by 12, 13 and 24% respectively. In addition, treatment with PAF (100 nM) or Ang1 (1 and 10 nM) at 24 hours also significantly decreased neutrophil necrosis by 45, 31 and 39% respectively (**Fig. 2C**). Since pro-survival activity of Ang1 and PAF on neutrophils was optimal at 24 hours, our experiments were subsequently performed at 24 hours post-isolation. The same experiments were performed in parallel with Ang2 (1 and 10 nM; 66 and 660 ng/ml). Again, we observed that Ang2 had no effect on neutrophil viability, apoptosis and necrosis from 0 to 24 hours post-isolation (**Fig. 2A-C**).

3.2 Ang1-mediated prosurvival activity is Tie2-dependent

In previous studies, we and other groups observed that angiopoietins could mediate their biological activities upon binding and activation of Tie2 receptor [22, 24, 65, 66]. Thus, we wanted to assess if the prosurvival activity of Ang1 is implying Tie2 participation. In the current set of experiments, neutrophils were pretreated with a goat polyclonal blocking anti-human Tie2 antibody (5, 10 or 20 □g/ml) or control goat IgG isotype (20 μg/ml) for 1 hour before stimulation with PBS or Ang1 (10 nM) for an additional 23 hours. Treatment with control goat IgG or with anti-Tie2 blocking antibodies (20 μg/ml) had no significant effect on basal neutrophil viability (**Fig. 3**). Pretreatment of neutrophils with anti-Tie2 blocking antibodies reduced in a concentration-dependent manner the prosurvival activity of Ang1 with a maximal effect being achieved at 20 μg/ml (84% inhibition) (**Fig. 3**).

Pretreatment of neutrophils with anti-Tie2 blocking antibodies did not reverse Ang1 mediated anti-apoptotic activity but did abrogate Ang1-anti-necrotic activity (**Fig. 3**).

3.3 Additive effect of PAF and Ang1 on neutrophil viability

Since PAF is a neutrophil pro-survival mediator [42], and as observed in our current study, we thus wanted to address whether both angiopoietins and PAF could cooperate on neutrophil viability. Neutrophils were treated with Ang1 (0.1 – 10 nM) in the presence or absence of PAF (100 nM) for 24 hours. Treatment with PAF or Ang1 (1 and 10 nM) increased neutrophil viability by 26, 23 and 36% respectively (**Fig. 4**). Combining PAF (100 nM) with Ang1 (1 and 10 nM) provided an additive effect, increasing by 48 and 60% the viability of the neutrophils (raising the basal neutrophil viability from 43.4 to 64.4 and 69.6%, respectively). Furthermore, the combination of PAF (100 nM) with Ang1 (1 and 10 nM) provided a significant increase of neutrophil survival as compared to the cells treated either with PAF (100 nM) or Ang1 (1 or 10 nM) alone (**Fig. 4**). Neutrophils were also treated with Ang1 (0.1 nM) to assess whether PAF (100 nM) could prime an effect of Ang1 at a concentration, which by itself does not induce neutrophil viability. This latter combination did not provide an additive effect on neutrophil viability (**Fig. 4**). In addition, treatment of neutrophils with PAF or Ang1 provided a significant decrease of apoptosis and their combination (PAF; 100 nM and Ang1; 10 nM) provided an additive capacity to decrease neutrophil apoptosis as compared

to the cells treated either with PAF (100 nM) or Ang1 (10 nM) alone (**Fig. 4**). Finally, the combination of PAF (100 nM) with Ang1 (1 and 10 nM) provided a significant decrease of neutrophil necrosis as compared to the cells treated either with PAF (100 nM) or Ang1 (1 or 10 nM) alone (**Fig. 4**).

3.4 Contribution of PAF on Ang1 pro-survival activity

In the current setting of experiments, we wanted to address if endogenous PAF is involved in Ang1 pro-survival activity on neutrophils. We thus pretreated neutrophils with a selective PAF receptor antagonist (BN 52021; 50 and 100 μM) [67, 68], or the corresponding control vehicle (DMSO) for 1 hour prior to the addition of PAF (100 nM) or Ang1 (10 nM) for an additional 23 hours. Treatment with BN 52021 alone did not modulate basal neutrophil viability. In pretreatment, BN 52021 abrogated completely PAF-mediated pro-survival activity, bringing the survival rate of the neutrophil even below to the control-PBS level (**Fig. 5**). In addition, a pretreatment with BN 52021 abrogated as well the PAF-mediated anti-apoptotic effect on neutrophils (**Fig. 5**). On the other hand, a pretreatment with BN 52021 did not modulate significantly the effect of Ang1 on neutrophil viability, apoptosis and necrosis (**Fig. 5**). We previously reported that in ECs, both angiopoietins induce PAF synthesis through the activation of $sPLA_2$-V and independently from $cPLA_2$ activation [21]. In function of the stimulus, both PLA_2s can be activated to promote neutrophil PAF synthesis [69-73]. We thus pretreated the neutrophils with a $sPLA_2$ inhibitor (scalaradial; up to 20 μM) [21, 74, 75], a

$cPLA_2$ inhibitor ($AACOCF_3$; up to 20 μM) [21, 74-76] or their corresponding control vehicles (DMSO or ethanol, respectively) for 1 hour prior to the addition of Ang1 (10 nM) for an additional 23 hours. Treatment with both inhibitors ($AACOCF_3$ and scalaradial) did not modulate Ang1 pro-survival activity on neutrophils (data not shown).

3.5 Additive effect of IL-8 and Ang1 on neutrophil viability

We reported that both angiopoietins can potentiate IL-8-induced neutrophil migration [24] and since IL-8 is a neutrophil pro-survival mediator [54], we wanted to assess if the angiopoietins and IL-8 could cooperate on neutrophil viability. First, we addressed the capacity of IL-8 to promote neutrophil viability. Upon a 24-hour incubation period, we observed a bell-shape concentration-dependent anti-apoptotic effect of IL-8 (5 – 200 nM) on neutrophil viability with an optimal effect being achieved at 25 nM, providing a 34% increase of neutrophil viability (raising the basal viability from 43.4 to 58.0%) (**suppl. data Fig. 2**). Neutrophils were then treated with Ang1 (0.1 – 10 nM) in presence or absence of IL-8 (25 nM) for 24 hours. The combination of IL-8 (25 nM) with Ang1 (10 nM) provided an additive effect, increasing basal neutrophil viability by 56%. This combination provided also a significant increase of neutrophil viability as compared to cells treated with Ang1 (10 nM) alone (**Fig. 6**). Neutrophils were also treated with Ang1 (0.1 nM) to assess whether IL-8 (25 nM) could prime an effect of Ang1 at a concentration, which by itself, does not provided a significant effect on neutrophil viability (**Fig.**

6). In addition, IL-8 or Ang1-treated neutrophils had a significant decrease of cell apoptosis, which was also significant upon the combination of IL-8 (25 nM) and Ang1 (10 nM) (**Fig. 6**). Finally, treatment of the neutrophils with IL-8 (25 nM) and Ang1 (10 nM), alone or combined, led to a significant decrease of cell necrosis (**Fig. 6**).

3.6 Contribution of IL-8 on Ang1 pro-survival activity

Both angiopoietins are known to promote similar pro-inflammatory activities in neutrophils [22, 24]. However, in our current set of experiments, we demonstrate differential activities of Ang1 and Ang2 on neutrophil viability. In addition, we recently observed that Ang1, but not Ang2, induces IL-8 synthesis and release from neutrophils in a concentration-dependent manner with an optimal effect at 10 nM and within 2 hours post-stimulation [77]. Therefore, we wanted to address whether IL-8 was implicated in Ang1-induced neutrophil viability. Neutrophils were pretreated with goat anti-human IL-8 blocking IgG antibodies (20 - 100 μg/ml) or control goat IgG isotype (100 μg/ml) for 2 hours before stimulation with PBS, IL-8 (25 nM) or Ang1 (10 nM) for an additional 22 hours. Treatment with control goat IgG or with anti-IL-8 blocking antibodies (100 μg/ml) had no effect on basal neutrophil viability (**Fig. 7**). Pretreatment with anti-IL-8 blocking antibodies reduced both IL-8 (25 nM) and Ang1 (10 nM)-induced neutrophil viability in a concentration-dependent manner with a maximal effect at 100 μg/ml for IL-8 (92% decrease) and 50 μg/ml for Ang1 (81% decrease) (**Fig. 7**). In addition, treatment

with IL-8 significantly decreased both apoptotic and necrotic neutrophils, whereas pretreatment with anti-IL-8 blocking antibodies (100 μg/ml) provided a trend to reverse the effect of IL-8 on neutrophil apoptosis, and significantly reversed the effect of IL-8 on neutrophil necrosis. Similarly, Ang1 also decreased significantly neutrophil apoptosis and necrosis, whereas pretreatment with anti-IL-8 blocking antibodies (50 and 100 μg/ml) significantly reversed only the effect of Ang1 on neutrophil necrosis while having a trend to reverse the effect of Ang1 on neutrophil apoptosis (**Fig. 7**).

3.7 Contribution of IL-8 on PAF pro-survival activity

Since Ang1 and PAF are both capable to promote IL-8 synthesis and release by the neutrophils [77], and that Ang1 pro-survival activity is IL-8-dependent, we thus wanted to address if PAF pro-survival activity is also IL-8-dependent. Neutrophils were pretreated with goat anti-human IL-8 blocking IgG antibodies (20 - 100 μg/ml) or control goat IgG isotype (100 μg/ml) for 2 hours before stimulation with PBS or PAF (100 nM) for an additional 22 hours. Treatment with control goat IgG or with anti-IL-8 blocking antibodies (100 μg/ml) had no effect on basal neutrophil viability (**Fig. 8**). Pretreatment with anti-IL-8 blocking antibodies (20, 50 and 100 μg/ml) reduced significantly PAF-induced neutrophil viability in a concentration-dependent manner with a maximal effect at 100 μg/ml (92% decrease) (**Fig. 8**). In addition, treatment with PAF significantly decreased both apoptotic and necrotic neutrophils, whereas pretreatment with anti-IL-8 blocking antibodies (20, 50 and

100 μg/ml) provided a trend to reverse the effect of PAF on neutrophil apoptosis, and significantly reversed the effect of PAF on neutrophil necrosis (**Fig. 8**).

3.8 Neutrophil chemotactic activity

We previously reported the capacity of Ang1 and IL-8 to promote neutrophil migration [24]. Herein, we wanted to ascertained if the neutrophils upon a 24-hour incubation period either under PBS or Ang1 treatment were still capable to migrate upon stimulation by pro-chemotactic mediators such as IL-8 and fMLP. On freshly isolated neutrophils, IL-8 (25 nM) [24] increased by 6.3-fold the migration of neutrophils upon a 60-minute incubation period. Similarly, a treatment with fMLP (0.1 – 10 μM) [78, 79] provided a bell-shape increase of neutrophil migration with a peak mediated at 1 μM (13.9-fold) **(Fig. 9)**. Upon a 24-hour incubation period in control media containing either control vehicle (PBS) or Ang1 (10 nM), we performed a 60-minute chemotactic assay and observed that the neutrophils maintained a similar capacity to respond to IL-8 (7.8- and 6.5-fold of increase respectively) and fMLP (1 μM; 9.3- and 8.1-fold of increase respectively) (**Fig. 9**).

4. DISCUSSION

In the present study, we report for the first time that upon Tie2 activation, Ang1 has a pro-survival activity on neutrophil, whereas Ang2 has no effect. The combination of known pro-inflammatory and pro-survival mediators for the neutrophils, namely PAF and IL-8, provided an additive effect on Ang1-mediated pro-survival activity. In addition, using a selective PAF receptor antagonist and blocking IL-8 antibodies, we delineated that Ang1 pro-survival activity is IL-8 dependent.

4.1 Differential effects of Ang1 and Ang2 on neutrophil viability

Ang1 and Ang2 have been reported to promote differential activities; Ang1 being recognized as a Tie2 receptor agonist, supporting the maturation and the stabilization of the neovessels, while Ang2 was recognized as an antagonist, promoting vascular permeability and the regression of pre-existing vasculature [3, 4, 14, 15]. However, under specific conditions, Ang2 can also act as a Tie2 agonist by supporting Tie2 phosphorylation and associated downstream biological activities [16-19]. For instance, in ECs, Ang1 is recognized to inhibit apoptosis through the activation of PI3K/Akt and ERK1/2 pathways, which cooperate to prevent pro-apoptotic caspase-3, -7, and -9 activation [10, 11]. For Ang2, the data are less clear; some groups reported that Ang2 has pro-apoptotic activities [55, 56], whereas others reported that Ang2 could promote ECs survival through the activation of ERK1/2 and PI3K pathways [17, 57]. In addition, we reported that both

angiopoietins share common agonistic pro-inflammatory activities, namely, on their capacity to promote endothelial PAF synthesis and P-selectin translocation [20-22]. In neutrophils, we reported the expression of Tie2 receptor, and the capacity of both angiopoietins to induce PAF synthesis, CD11/CD18 (β_2-integrin) activation, neutrophil adhesion and migration [22, 24]. This latter effect was also confirmed by an independent group [65]. In addition, considering that both angiopoietins can modulate ECs viability and induce the synthesis and release of a neutrophil pro-survival mediator, namely PAF [10, 11, 17, 22, 42, 55-57], we were led to address the capacity of both angiopoietins to modulate neutrophil viability, and if so, define the role of PAF in that process.

In the current study, we performed a concentration- and time-dependent assay, and observed that Ang1 is providing a pro-survival activity on the neutrophils by delaying the entry of cells into apoptotic and necrotic phase, while Ang2 had no significant effect on basal viability. In addition, we observed that Ang1/Tie2 interaction is needed to support Ang1 pro-survival activity, as a pretreatment with selective blocking anti-human Tie2 antibodies, almost completely abrogated (≈85%) Ang1 pro-survival activity in neutrophils. The residual effect could be due to an incomplete blockade of Tie2 receptor by the selective antibodies, thus, allowing partial Ang1/Tie2 interaction and biological activity. Another possibility could be that Ang1 can induce some biological activities upon its binding to specific integrins, independently from Tie2 participation [9, 80, 81]. If so, then it appears that Ang1/integrins interaction would have a minor contribution on Ang1 pro-survival activity.

4.2 Cooperation and contribution of PAF and IL-8 on Ang1-mediated pro-survival activity

In addition to the differential capacity of Ang1 to prolong the viability of the neutrophils, as opposed to Ang2, we recently observed another differential effect between Ang1 and Ang2 on neutrophils. Indeed, we reported that Ang1 (but not Ang2) promotes IL-8 synthesis and release from neutrophils [77]. Since IL-8 is a pro-survival mediator for the neutrophils [54], we hypothesized that the synthesis and/or the release of IL-8, might as well, like endogenous PAF supports the pro-survival activity of Ang1 on neutrophils. Our data demonstrate that a combination of PAF or IL-8 with Ang1 produced an additive effect on neutrophil viability. These results are in agreement with previous studies demonstrating that under *in vivo* pro-inflammatory environments, different mediators can cooperate to increase neutrophil viability, thereby delaying their entry into apoptotic phase, even up to several days [26] and thus, resulting in an enhanced inflammatory state which can become pathological [29].

To delineate the contribution of PAF on Ang1 pro-survival activity in neutrophils, we addressed the effect of a selective PAF receptor antagonist (BN 52021) [67, 68] on Ang1 anti-apoptotic activity. Treatment with BN 52021 did not modulate basal neutrophil viability, whereas it was efficient to prevent pro-survival activity mediated by exogenous PAF. In addition, in pretreatment, BN 52021 did not inhibit Ang1 pro-survival activity on neutrophils, thus, suggesting that Ang1 anti-apoptotic activity is PAF-independent. We previously reported that Ang1 and Ang2 could induce PAF synthesis from ECs through the activation of $sPLA_2$-V,

and independently from $cPLA_2$ participation [21]. We also observed that both angiopoietins could promote neutrophil PAF synthesis [22]. In neutrophils, both $cPLA_2$ and $sPLA_2$-V are expressed and can contribute to PAF synthesis in function of the stimuli [69, 71-73]. Thereby, we addressed the effect of selective inhibitors of the secreted and cytosolic form of PLA_2 on Ang1-mediated pro-survival activity on neutrophils. Pretreatment of neutrophils either with the $cPLA_2$ inhibitor [74-76], $AACOCF_3$, or the $sPLA_2$ inhibitor [74, 75, 82], scalaradial, did not reduced Ang1 pro-survival activity on neutrophils. Consequently, if both angiopoietins (Ang1 and Ang2) can support the endogenous PAF synthesis from neutrophils [22], the concentration of PAF synthesized is not sufficient to participate to Ang1-mediated pro-survival activity on the neutrophils.

Since PAF cannot be considered as the pro-inflammatory mediator supporting Ang1-mediated pro-survival activity, we thus addressed whether IL-8 release was implicated in Ang1-induced neutrophil viability. Under basal condition, there is no or marginal release of IL-8 by the neutrophils, and a treatment with an anti-human IL-8 blocking antibodies did not modulate the basal survival rate. However, in pretreatment to Ang1-stimulation, the anti-IL-8 blocking antibodies provided a marked reduction (≈80%) of Ang1 pro-survival effect. Consequently, these data are supporting our hypothesis that the synthesis and release of IL-8 plays a pivotal role as a discriminating factor for the differential capacity of Ang1, as opposed to Ang2, to support neutrophil viability. The incomplete reduction of Ang1-mediated pro-survival activity following a pretreatment with IL-8 blocking antibodies could suggest that Ang1 can activate

downstream signaling pathways capable to provide pro-survival/anti-apoptotic activities, and/or that Ang1 is capable to promote the release of other pro-survival mediators.

We also reported that PAF is capable to promote IL-8 synthesis and release by the neutrophils [77]. Since a treatment with exogenous PAF is capable to promote neutrophil viability, we addressed the contribution of IL-8 on PAF pro-survival activity. By pretreating the neutrophils with anti-IL-8 blocking antibodies, we observed an obliteration of PAF anti-apoptotic activity. These latter observations demonstrate that the synthesis and release of IL-8 upon a stimulation of neutrophils either with exogenous Ang1 or PAF is the common factor responsible for their pro-survival activity.

It is well defined that transvascular migrated neutrophils are playing a major role in the development of pathological inflammation by prolonging their survival half-life, thus, extending their capacity to promote the synthesis and release of pro-inflammatory mediators, cytokines and metaloproteinases [28, 29, 83-85]. We and other groups reported the chemotactic activity of Ang1 and other cytokines on freshly isolated neutrophils [24, 65, 86]. Herein, we observed that a treatment of the neutrophils with Ang1 under acute or prolong time period did not altered the capacity of the neutrophils to migrate upon their stimulation by chemotactic mediators, such as fMLP and IL-8.

Previous studies reported that under *in vitro* conditions, Ang1 possesses anti-inflammatory properties. Ang1 reduces VEGF-induced leukocyte adhesion

onto HUVEC and E-selectin expression [87] and thrombin-mediated neutrophil adhesion onto EC [88]. Other studies reported that under *in vivo* conditions, Ang1 has an anti-inflammatory role as observed in acute lung injury, endotoxic shock and cardiac remodeling [12, 13, 89]. Similarly, the genetic overexpression or overexpression following infection with adenovirus carrying the Ang1 gene did protect the vasculature from VEGF and irritant-induced leakage in mice [4, 90]. On the other hand, we and other groups reported that under *in vitro* conditions Ang1 can have pro-inflammatory activities [20-22, 24, 65, 77, 91]. In addition, it has also been reported that the administration of Ang1 did not inhibit VEGF-induced vascular permeability but delayed edema resolution, thus suggesting that Ang1 is not a universal inhibitor of EC permeability as previously thought [18]. Together, these observations could suggest that Ang1 may have differential biological activities in function of the biological conditions and environment.

In summary, our study is the first one to report that Ang1, upon its binding to Tie2 receptor, is providing a pro-survival activity on neutrophils, as opposed to Ang2, which has no effect. In addition, Ang1 prosurvival activity is mainly driven by its capacity to promote the synthesis and release of IL-8 by the neutrophils. However, additional studies will be needed to delineate which intracellular signaling pathways are participating in the induction of Ang1 pro-survival and anti-apoptotic activities on neutrophils. Together, our data provides novel insight in the capacity of Ang1 to prolong neutrophil viability and to promote the release of pro-inflammatory mediators, which could contribute to support the development of pathological vascular diseases associated to inflammatory response.

ABBREVIATIONS

Ang1: angiopoietin-1

Ang2: angiopoietin-2

ARDS: acute respiratory distress syndrome

$cPLA_2$: cytosolic phospholipase A_2

EC: endothelial cell

FITC: fluorescein isothiocyanate

fMLP: formyl-Methionyl-Leucyl-Phenylalanine

IL-8: interleukin-8

MAPK: mitogen-activated protein kinase

PAF: platelet activating factor

P.I.: propidium iodide

PKC: protein kinase C

SIRS: systemic inflammatory response syndrome

$sPLA_2$: secreted phospholipase A_2

TNF-α: tumor necrosis factor-α

VEGF: vascular endothelial growth factor

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6. FIGURE LEGENDS

Figure 1. Effect of angiopoietins on neutrophil viability in a concentration-dependent manner. Human neutrophils were incubated with PAF (100 nM; positive control), Ang1 (0.001 – 50 nM) (A) and Ang2 (0.001 – 50 nM) (B) for 24 hours at 37°C. Neutrophils were considered viable in absence of positive staining for both markers (annexin-V and P.I.; annexin-V^-/$P.I.^-$). Neutrophils were considered under apoptotic state when they stained positively for annexin-V and negatively for P.I. (annexin-V^+/$P.I.^-$). Neutrophils were considered necrotic when they stained positively for both markers (annexin-V^+/$P.I.^+$). *p<0.05; **p<0.01 and ***p<0.001 as compared to PBS-treated cells.

Figure 2. Effect of Ang1 and Ang2 on neutrophil viability in a time-dependent manner. Neutrophils were incubated with Ang1 (1 – 10 nM), Ang2 (1 – 10 nM) and PAF (100 nM) from 0 to 24 hours at 37°C. (A) Viable neutrophils (B) Apoptotic neutrophils and (C) Necrotic neutrophils. *p<0.05; **p<0.01 and ***p<0.001 as compared to PBS-treated cells.

Figure 3. Blocking anti-Tie2 antibodies reduce Ang1-mediated pro-survival effect on neutrophils. Neutrophils were pretreated with or without blocking anti-human Tie-2 IgG antibodies (5 – 20 μg/ml) for 1 hour. Then, neutrophils were incubated with or without Ang1 (10 nM) for 23 hours at 37°C. *p<0.05; **p<0.01;

***p<0.001 as compared to PBS-treated cells; $^{\lozenge}$p<0.05 as compared to Ang1-treated cells (10 nM).

Figure 4. Combination of PAF and Ang1 provides an additive effect on neutrophil viability. Neutrophils were incubated with Ang1 (0.1 – 10 nM) in presence or absence of PAF (100 nM) for 24 hours at 37°C. *p<0.05; **p<0.01; ***p<0.001 as compared to PBS-treated cells; $^{\lozenge}$p<0.05; $^{\lozenge\lozenge}$p<0.01; $^{\lozenge\lozenge\lozenge}$p<0.001 as compared to Ang1-treated cells (0.1, 1 and 10 nM); †p<0.05; ††p<0.01 and †††p<0.001 as compared to PAF-treated cells (100 nM).

Figure 5. BN 52021, a PAF receptor antagonist, did not reduce Ang1-mediated pro-survival effect on neutrophils. Neutrophils were pretreated with or without BN 52021 (50 or 100 μM) or with DMSO (2 μL; concentration used to dissolve BN 52021) for 1 hour. Then, neutrophils were incubated with or without PAF (100 nM) and/or Ang1 (10 nM) for 24 hours at 37°C. *p<0.05; **p<0.01; ***p<0.001 as compared to PBS-treated cells; ††p<0.01 as compared to PAF-treated cells (100 nM).

Figure 6. Combination of IL-8 and Ang1 provides an additive effect on neutrophil viability. Neutrophils were incubated with Ang1 (0.1 – 10 nM) in presence or absence of IL-8 (25 nM) for 24 hours at 37°C. *p<0.05; **p<0.01;

***p<0.001 as compared to PBS-treated cells; $^{\lozenge}$p<0.05 as compared to Ang1-treated cells (0.1 and 10 nM).

Figure 7. Blocking anti-IL-8 antibodies reduce Ang1-mediated pro-survival effect on neutrophils. Neutrophils were pretreated with or without blocking anti-human IL-8 IgG antibodies (20 – 100 μg/ml) for 2 hours. Then, neutrophils were incubated with or without IL-8 (25 nM) or Ang1 (10 nM) for 22 hours at 37°C. *p<0.05; **p<0.01; ***p<0.001 as compared to PBS-treated cells; $^{\lozenge}$p<0.05; $^{\lozenge\lozenge}$p<0.01; $^{\lozenge\lozenge\lozenge}$p<0.001 as compared to Ang1-treated cells (10 nM); †p<0.05 and ††p<0.01 as compared to IL-8-treated cells (25 nM).

Figure 8. Blocking anti-IL-8 antibodies reduce PAF-mediated pro-survival effect on neutrophils. Neutrophils were pretreated with or without blocking anti-human IL-8 IgG antibodies (20 – 100 μg/ml) for 2 hours. Then, neutrophils were incubated with or without PAF (100 nM) for 22 hours at 37°C. *p<0.05 and ***p<0.001 as compared to PBS-treated cells; $^{\lozenge}$p<0.05; $^{\lozenge\lozenge}$p<0.01; $^{\lozenge\lozenge\lozenge}$p<0.001 as compared to PAF-treated cells.

Figure 9. Neutrophil chemotactic activity is maintained upon 24-hour post-isolation period.

In vitro chemotactic assays were performed in Transwell-96 well permeable supports. The upper and lower parts of the wells were separated by a porous polycarbonate membrane filter (3 □m pore size). Briefly, the bottom wells were loaded with RPMI containing PBS, IL-8 (25 nM) or fMLP (0.1 – 10 □M). The upper wells were loaded with RPMI containing neutrophils. In function of the experiments, neutrophils were freshly added post-isolation (left panel) or pretreated for 24 hours with PBS or Ang1 (10 nM) (middle and right panels respectively) prior to a 1-hr chemotactic assay. At the end of the incubation period, the supernatant from the bottom wells was collected, and a count of migrating neutrophils was assessed with an Amnis ImageStream system. *$p<0.05$; **$p<0.01$ as compared to corresponding PBS-treated cells.

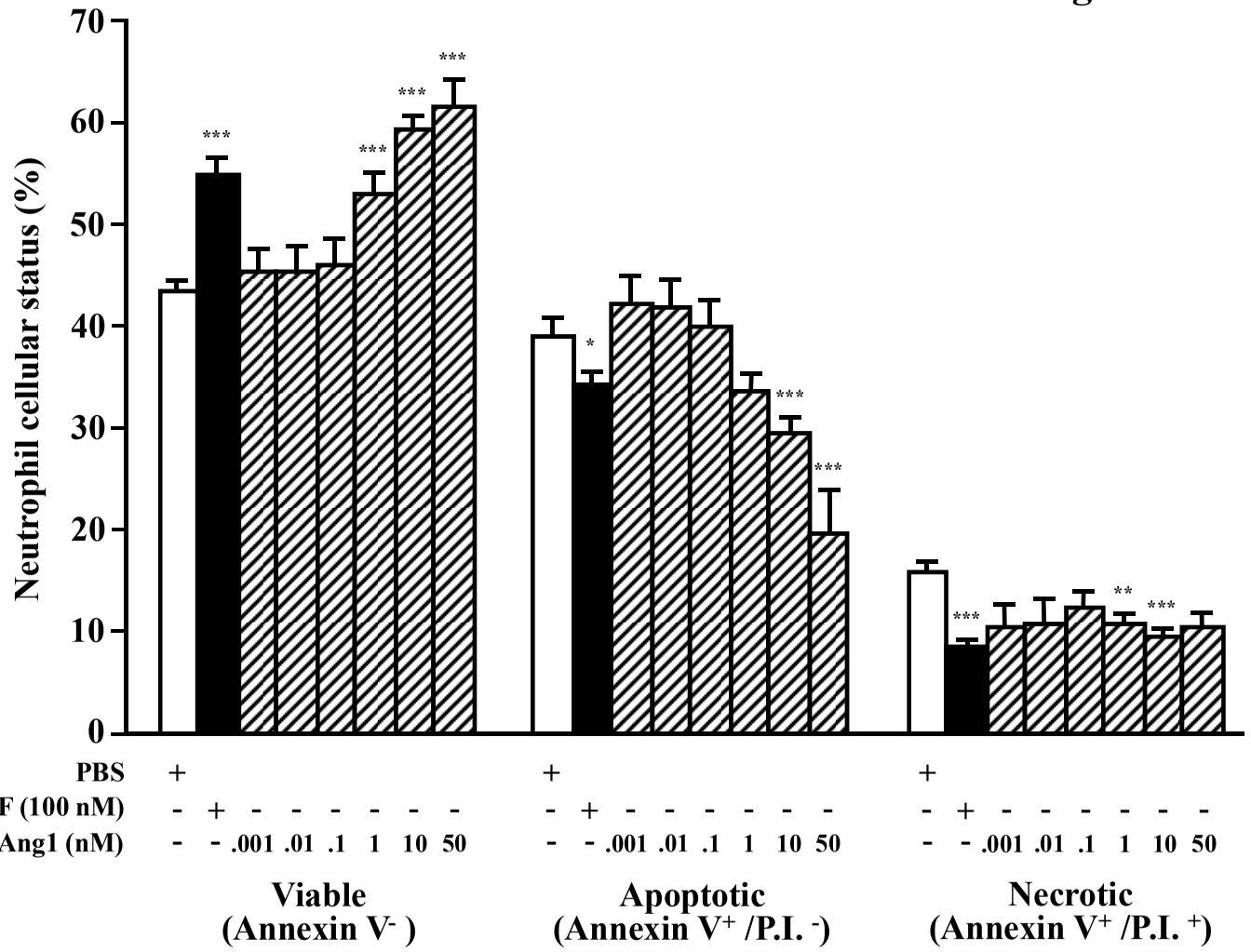
7. SUPPLEMENTAL DATA

Figure 1. Effect of PAF on neutrophil viability in a concentration-dependent manner. Human neutrophils were incubated with PAF (1 – 10 000 nM) for 24 hours at 37°C. *p<0.05 and ***p<0.001 as compared to PBS-treated cells.

Figure 2. Effect of IL-8 on neutrophil viability in a concentration-dependent manner. Human neutrophils were incubated with IL-8 (5 – 200 nM) for 24 hours at 37°C. *p<0.05 and ***p<0.001 as compared to PBS-treated cells.

A

Figure 1A


Neutrophil cellular status (%)
70
60
50
40
30
20
10
0
PBS + + + + + + + + + + + + + + + + + +
PAF (100 nM) - + - - - - - - - + - - - - - - - - - + - - - - - - - -
Ang1 (nM) - - .001 .01 .1 1 10 50 - - .001 .01 .1 1 10 50 - - .001 .01 .1 1 10 50
Viable (Annexin V-)
Apoptotic (Annexin V+ /P.I. -)
Necrotic (Annexin V+ /P.I. +)

B

Figure 1B

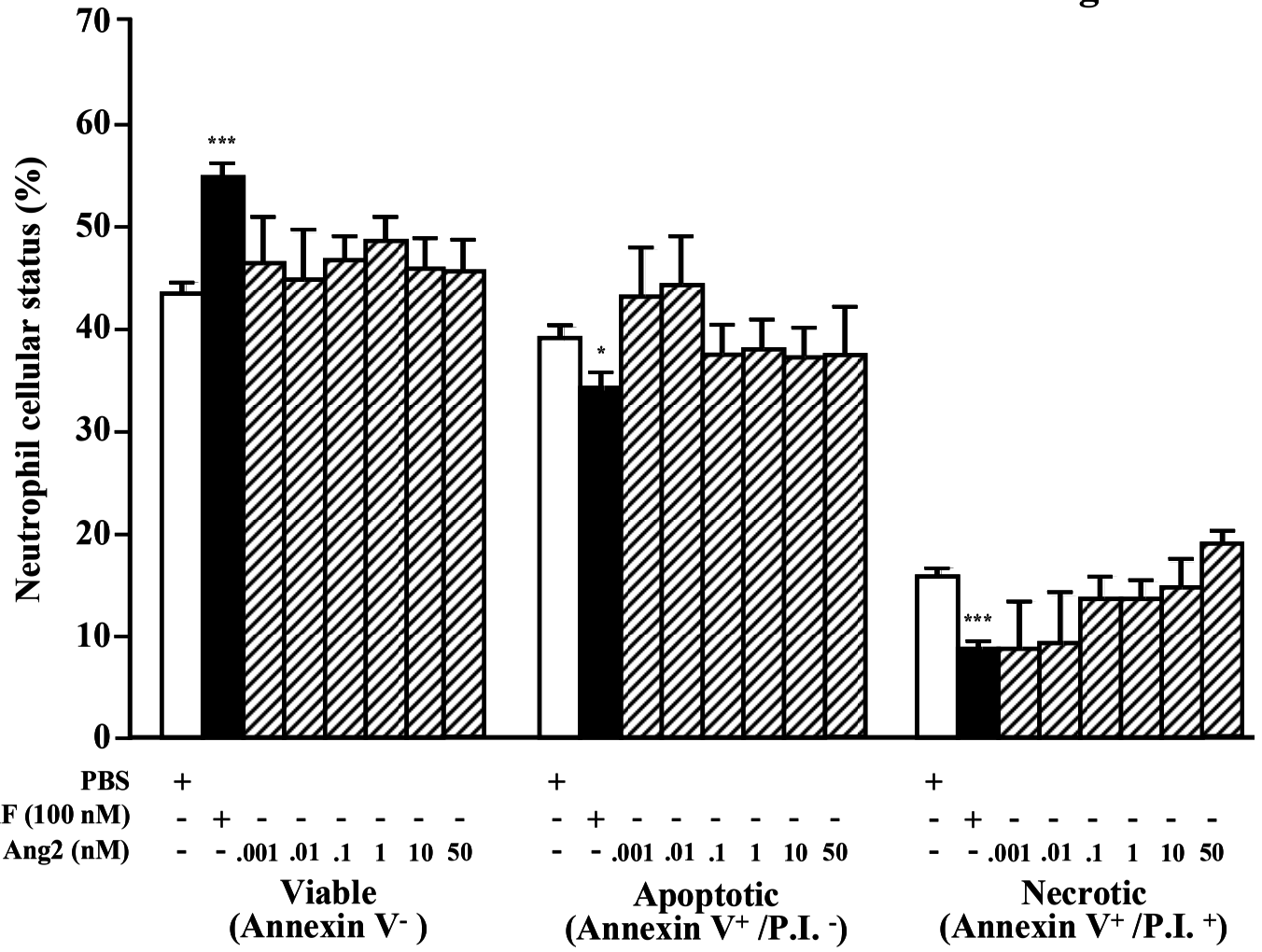

Neutrophil cellular status (%)
70
60
50
40
30
20
10
0
***
*
***
PBS + + + +
PAF (100 nM) - + - - - - - - - + - - - - - - - + - - - - - -
Ang2 (nM) - - .001 .01 .1 1 10 50 - - .001 .01 .1 1 10 50 - - .001 .01 .1 1 10 50
Viable (Annexin V⁻)
Apoptotic (Annexin V⁺ /P.I. ⁻)
Necrotic (Annexin V⁺ /P.I. ⁺)

Figure 2A

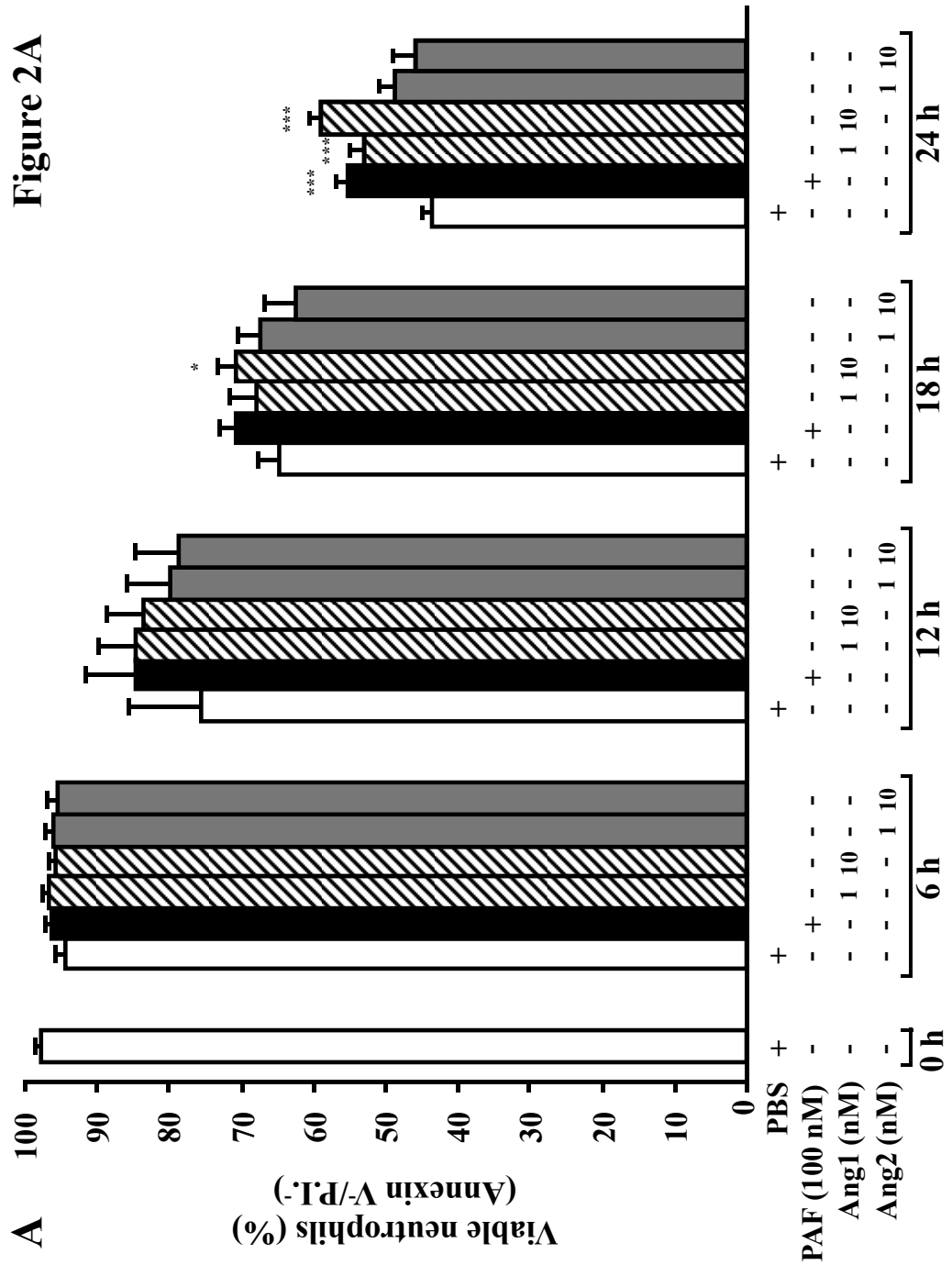
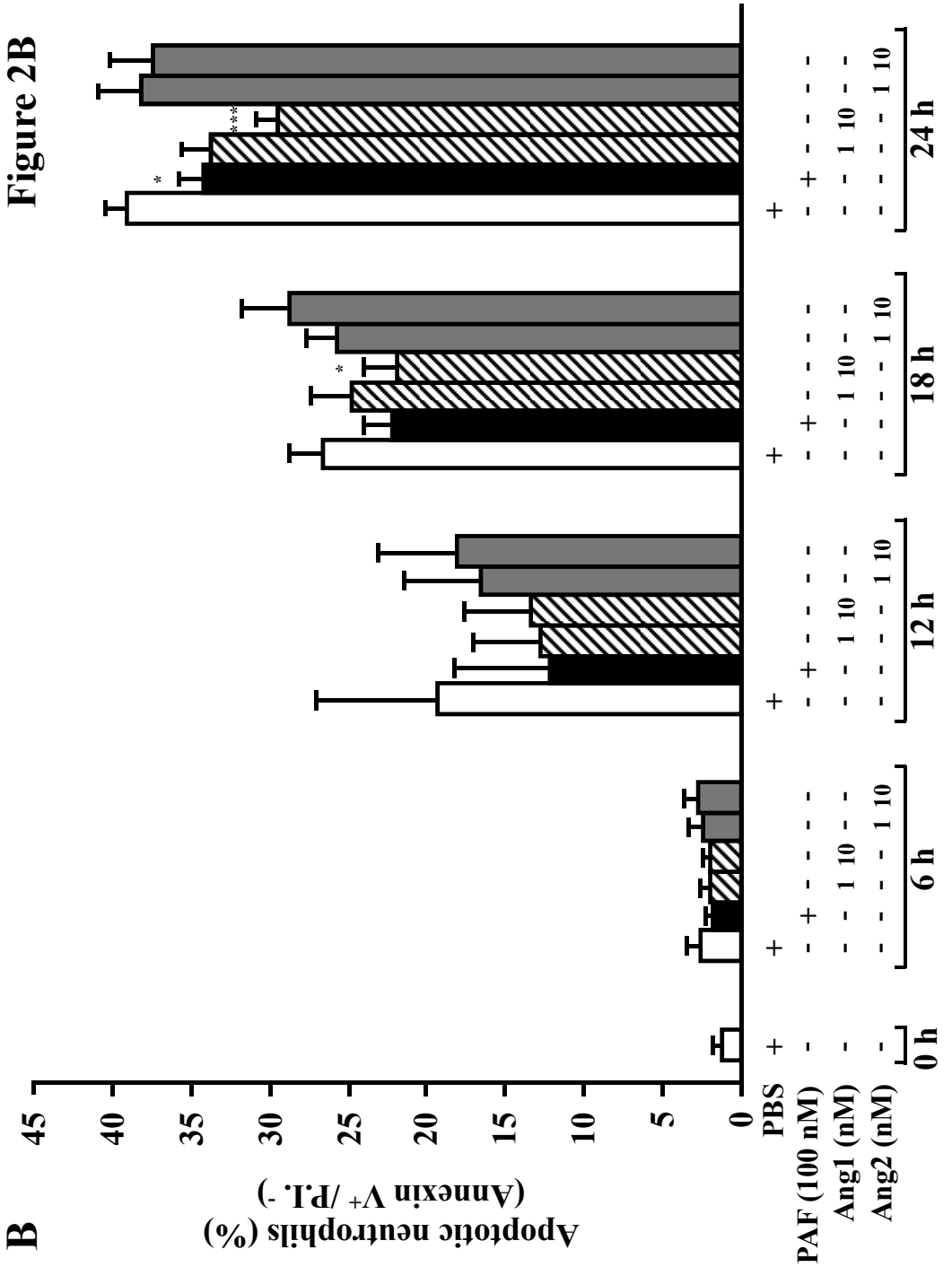

A
Viable neutrophils (%)
(Annexin V-/P.I.-)
100
90
80
70
60
50
40
30
20
10
0
*
***
***
***
PBS + + + + + + + + + + + + + + + + + +
PAF (100 nM) - - + - - - - - - + - - - - - - - - -
Ang1 (nM) - - - 1 10 - - - 1 10 - - - 1 10 - - -
Ang2 (nM) - - - - - 1 10 - - - - 1 10 - - - - 1 10
0 h
6 h
12 h
18 h
24 h

Figure 2B


B
Apoptotic neutrophils (%)
(Annexin V+ /P.I. -)
45
40
35
30
25
20
15
10
5
0
PBS + + + + + + + + + + + +
PAF (100 nM) - - + - - - - + - - - -
Ang1 (nM) - - - 1 10 - - - 1 10 - -
Ang2 (nM) - - - - - 1 10 - - - 1 10
0 h
6 h
12 h
18 h
24 h
*
*
*
***

C

Figure 2C

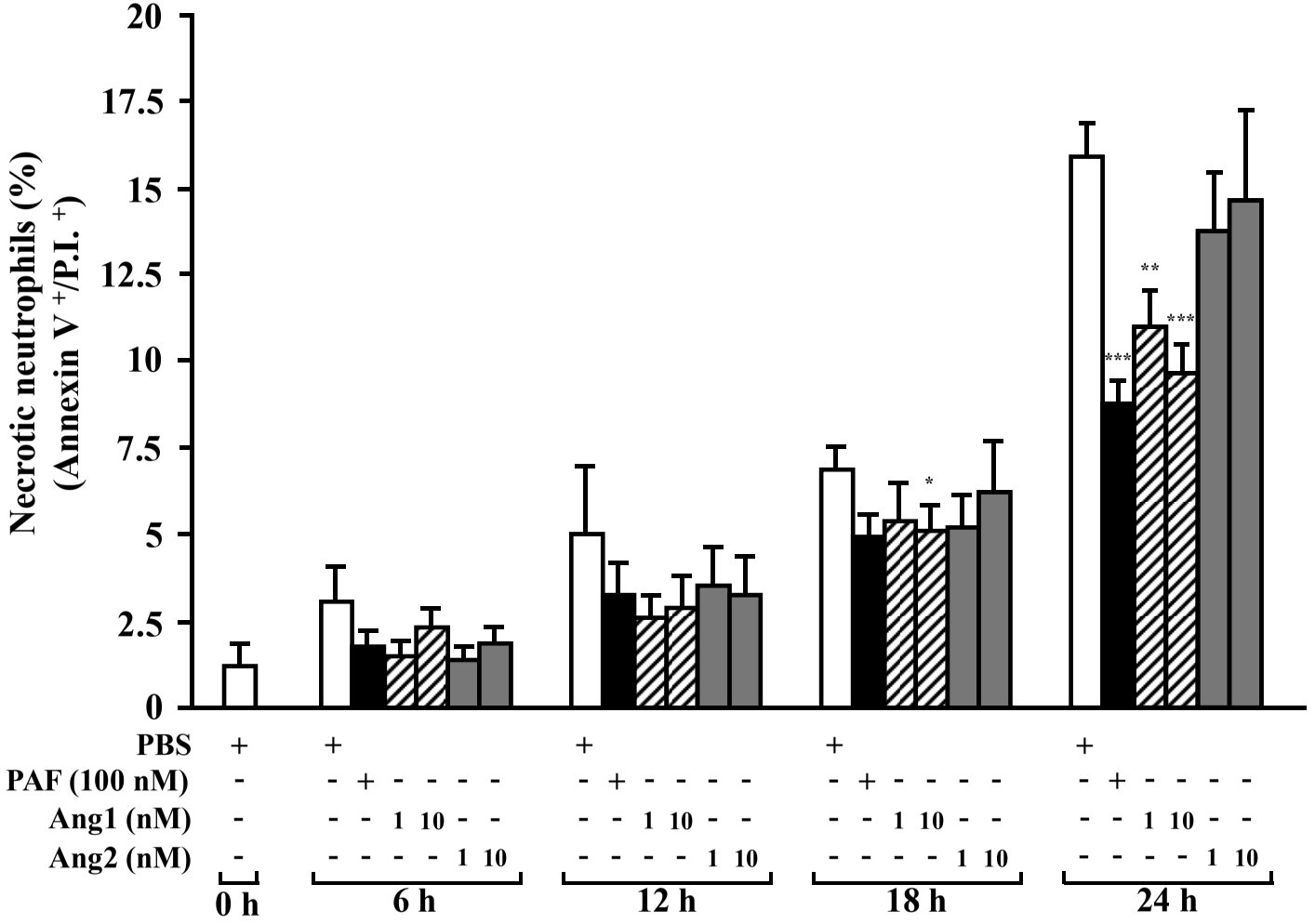

Necrotic neutrophils (%)
(Annexin V +/P.I. +)
20
17.5
15
12.5
10
7.5
5
2.5
0
***
**
***
*
PBS + + + + +
PAF (100 nM) - - + - - - - - + - - - - - + - - - - - + - - - -
Ang1 (nM) - - - 1 10 - - - - 1 10 - - - - 1 10 - - - - 1 10 -
Ang2 (nM) - - - - - 1 10 - - - - 1 10 - - - - 1 10 - - - - 1 10
0 h
6 h
12 h
18 h
24 h

Figure 3

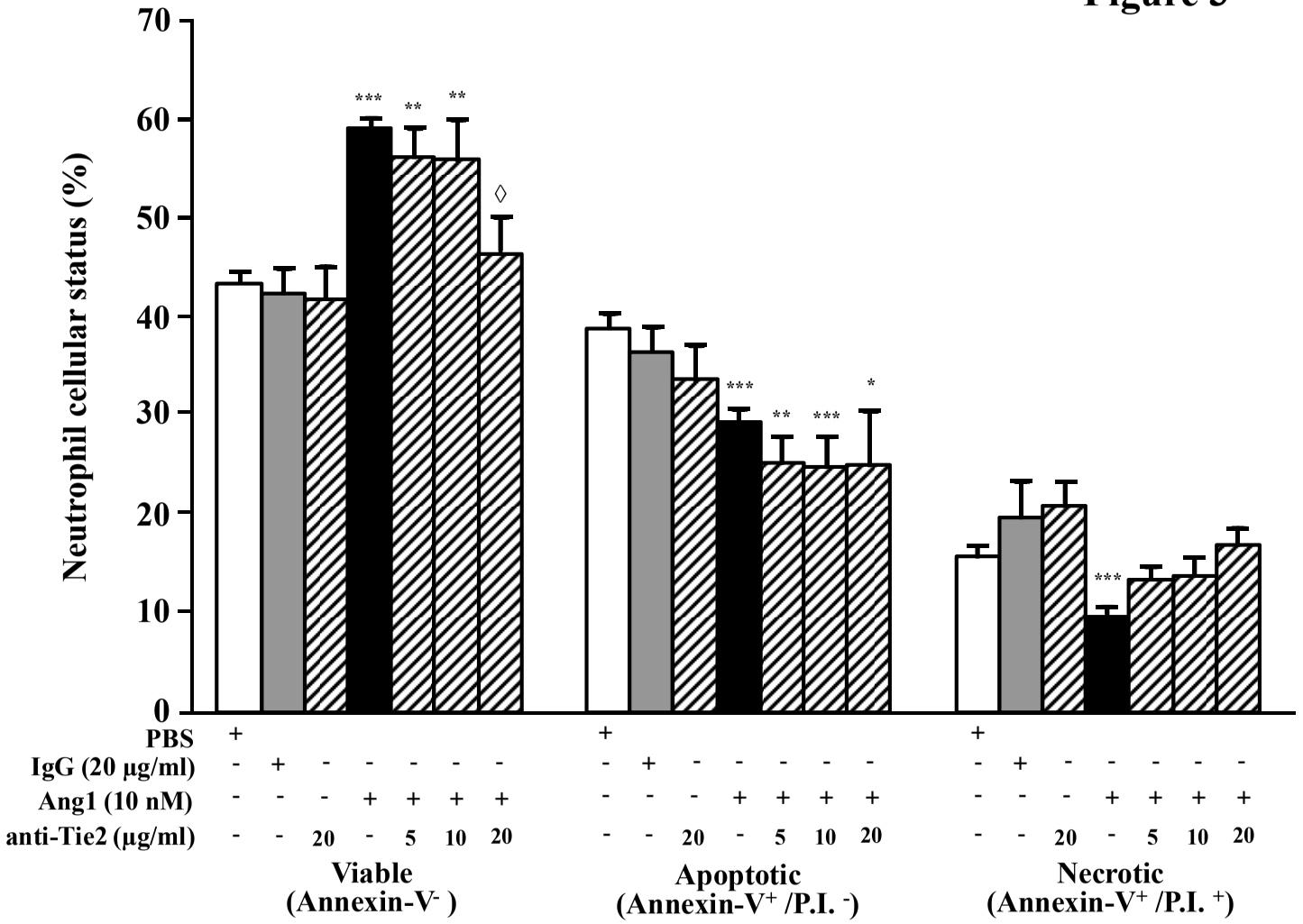

Neutrophil cellular status (%)
70
60
50
40
30
20
10
0
***
**
**
◊
***
**
***
*
***
PBS +
IgG (20 µg/ml) - + - - - - - - + - - - - - - + - - - - - -
Ang1 (10 nM) - - - + + + + - - - + + + + - - - + + + +
anti-Tie2 (µg/ml) - - 20 - 5 10 20 - - 20 - 5 10 20 - - 20 - 5 10 20
Viable (Annexin-V-)
Apoptotic (Annexin-V+ /P.I. -)
Necrotic (Annexin-V+ /P.I. +)

Figure 4

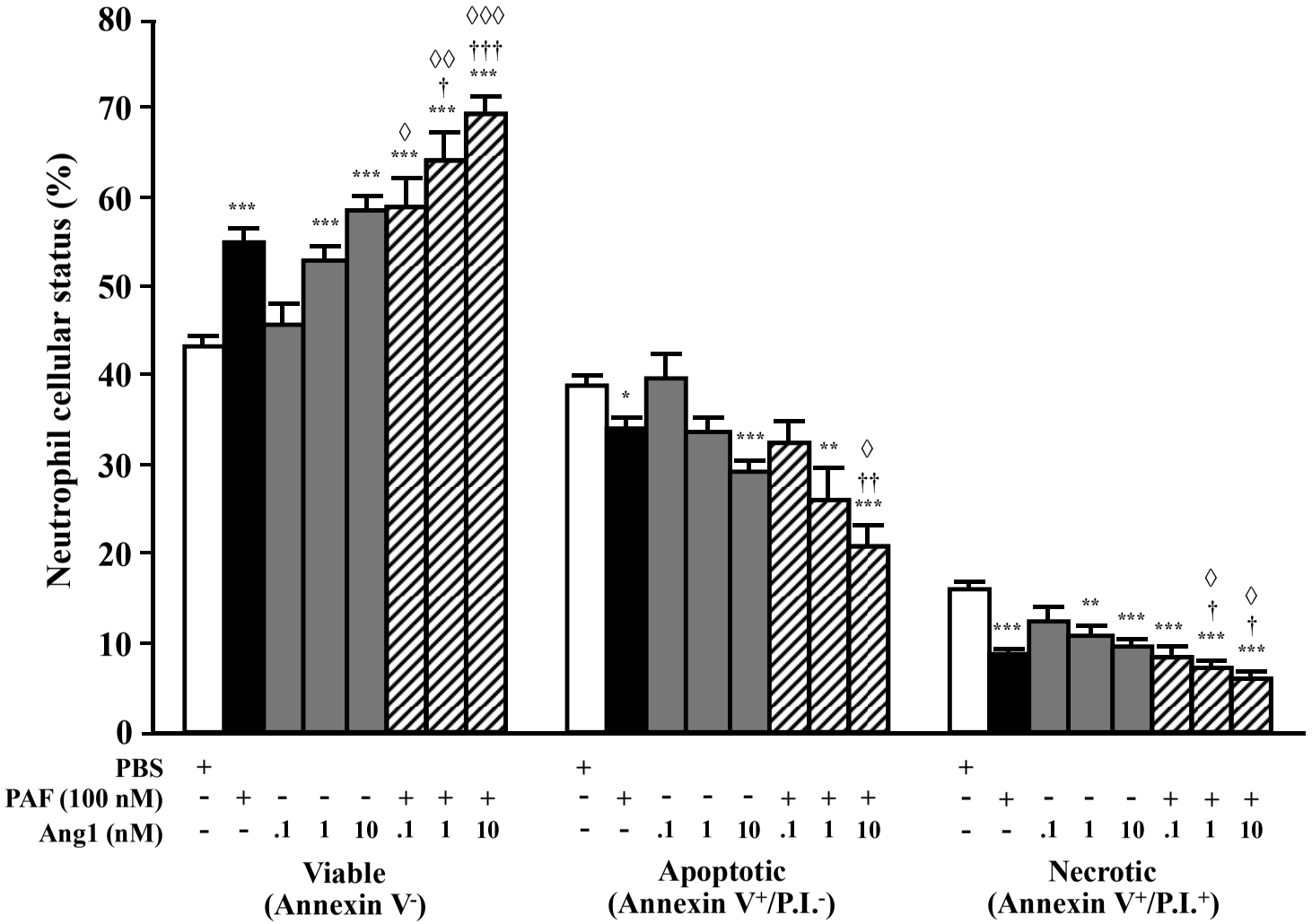

Neutrophil cellular status (%)
80
70
60
50
40
30
20
10
0
PBS + + +
PAF (100 nM) - + - - - + + + - + - - - + + + - + - - - + + +
Ang1 (nM) - - .1 1 10 .1 1 10 - - .1 1 10 .1 1 10 - - .1 1 10 .1 1 10
Viable (Annexin V-)
Apoptotic (Annexin V+/P.I.-)
Necrotic (Annexin V+/P.I.+)

Figure 5

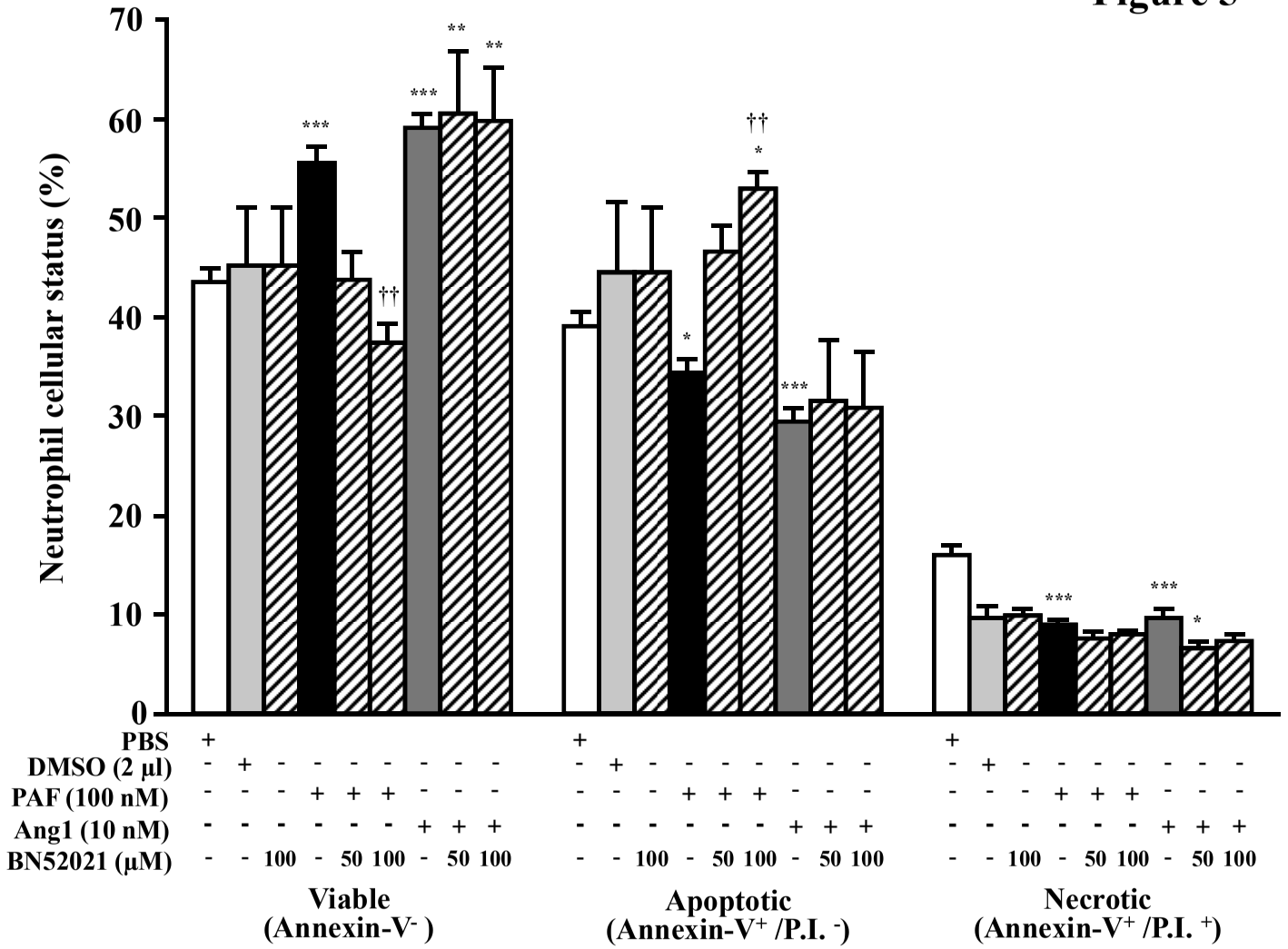

Neutrophil cellular status (%)
70
60
50
40
30
20
10
0
PBS
DMSO (2 µl)
PAF (100 nM)
Ang1 (10 nM)
BN52021 (µM)
Viable
(Annexin-V-)
Apoptotic
(Annexin-V+ /P.I. -)
Necrotic
(Annexin-V+ /P.I. +)

Figure 6

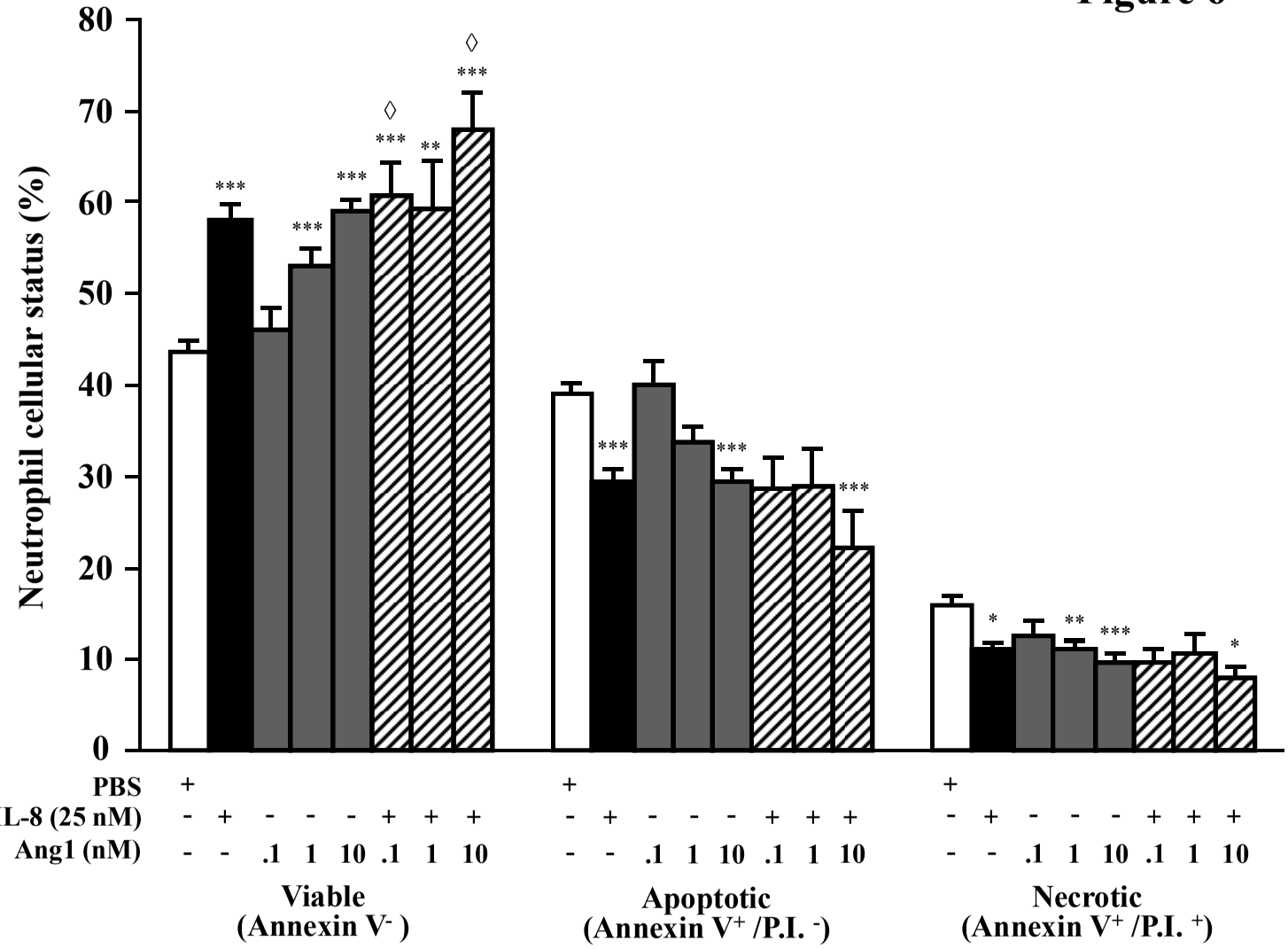

Neutrophil cellular status (%)
80
70
60
50
40
30
20
10
0
PBS + + +
IL-8 (25 nM) - + - - - + + + - + - - - + + + - + - - - + + +
Ang1 (nM) - - .1 1 10 .1 1 10 - - .1 1 10 .1 1 10 - - .1 1 10 .1 1 10
Viable (Annexin V-)
Apoptotic (Annexin V+ /P.I. -)
Necrotic (Annexin V+ /P.I. +)

Figure 7

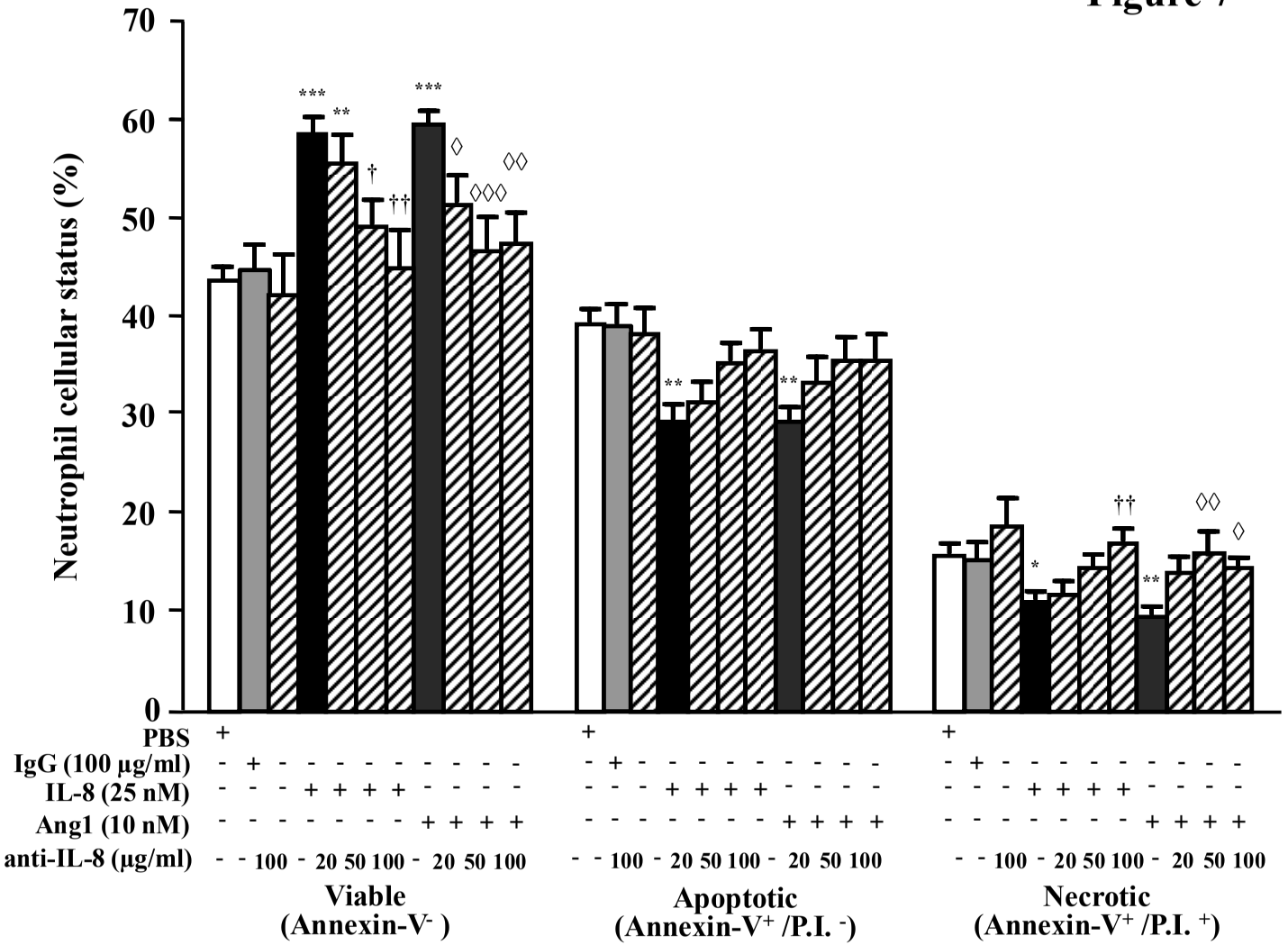

Neutrophil cellular status (%)
70
60
50
40
30
20
10
0
PBS
IgG (100 µg/ml)
IL-8 (25 nM)
Ang1 (10 nM)
anti-IL-8 (µg/ml)
100 20 50 100 20 50 100
Viable
(Annexin-V-)
Apoptotic
(Annexin-V+ /P.I. -)
Necrotic
(Annexin-V+ /P.I. +)

Figure 8

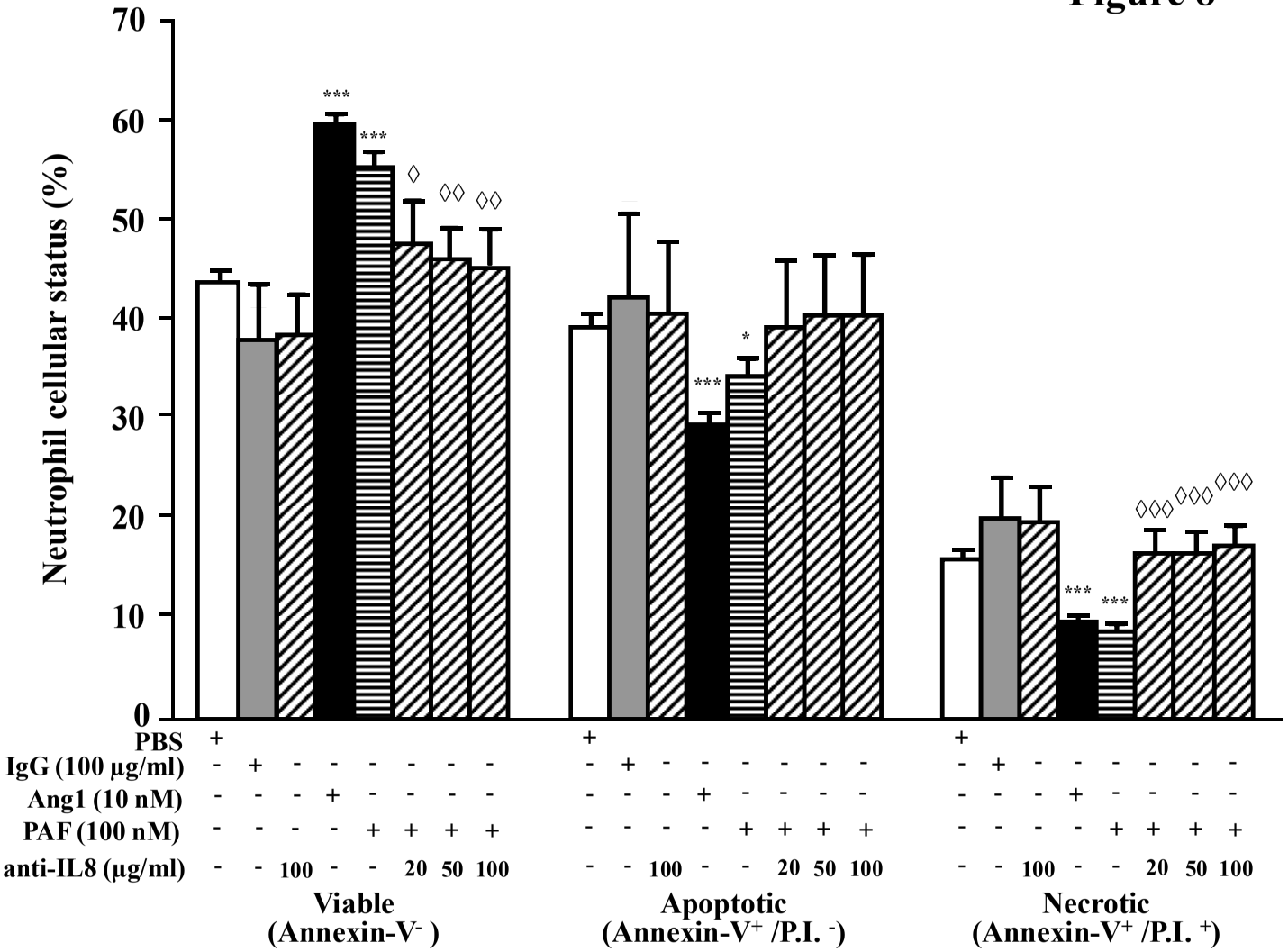
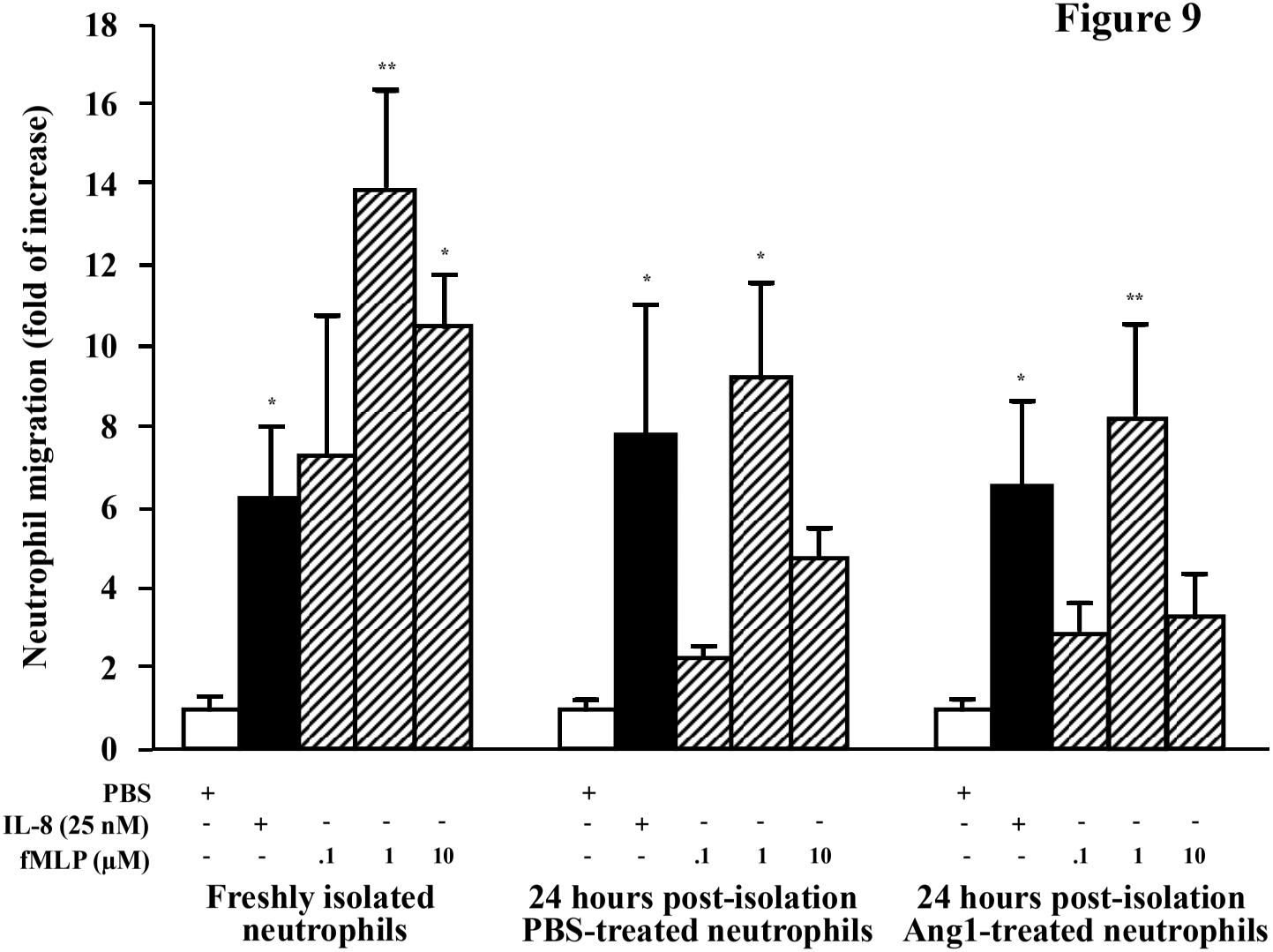
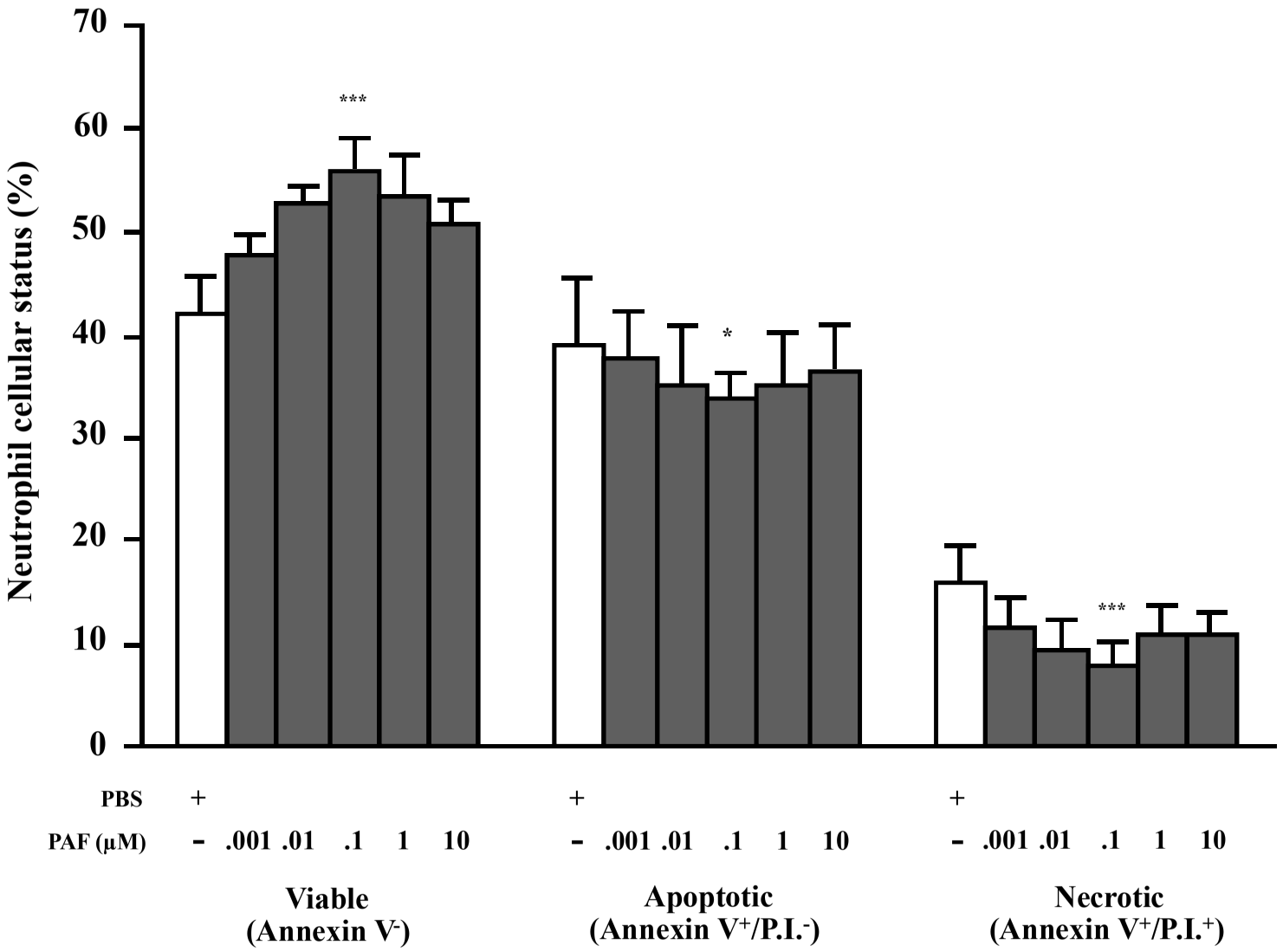
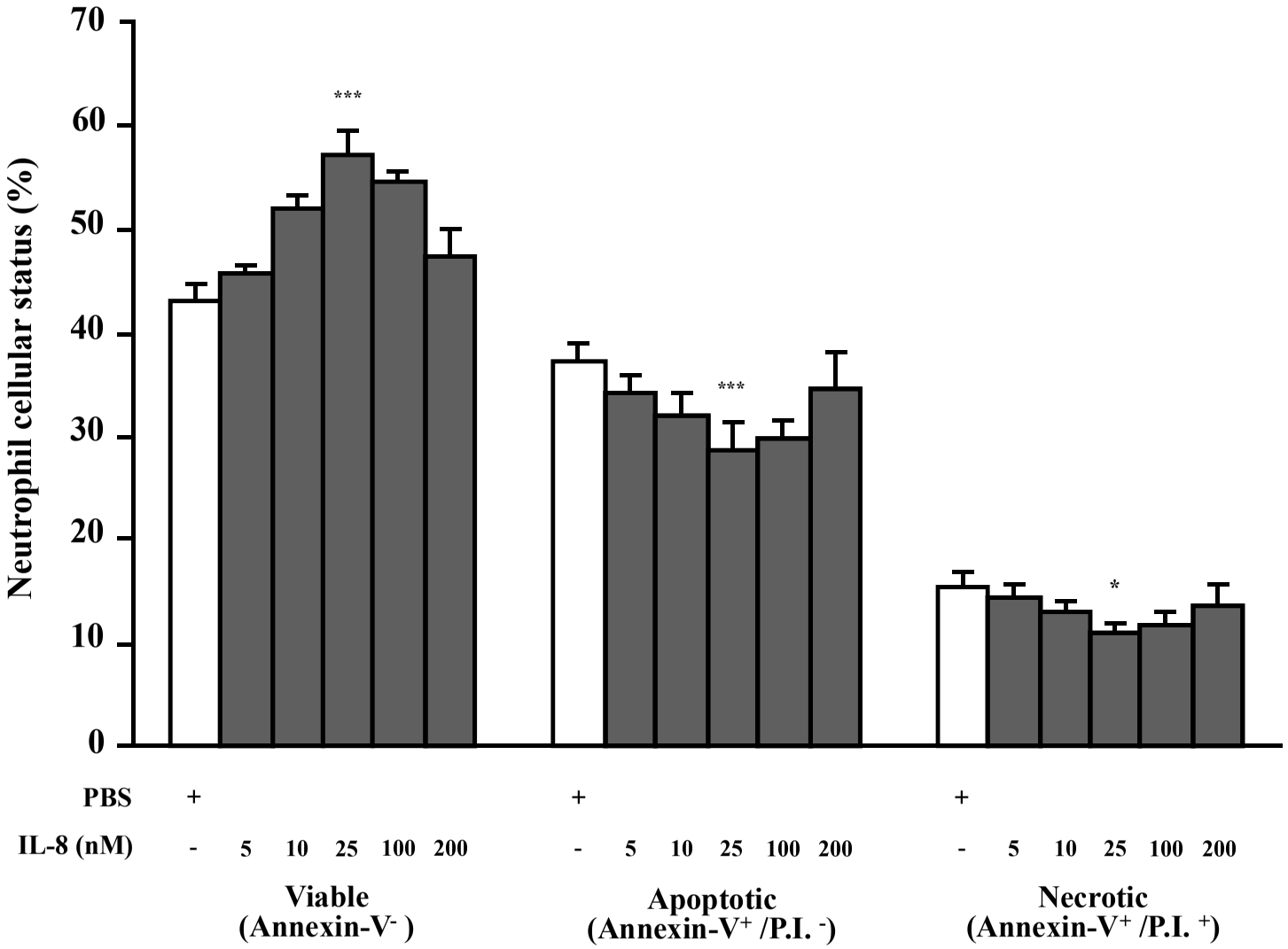

Neutrophil cellular status (%)
70
60
50
40
30
20
10
0
***
***
◊
◊◊
◊◊
***
*
***
***
◊◊◊
◊◊◊
◊◊◊
PBS
IgG (100 µg/ml)
Ang1 (10 nM)
PAF (100 nM)
anti-IL8 (µg/ml)
100
20
50
100
Viable
(Annexin-V-)
Apoptotic
(Annexin-V+ /P.I. -)
Necrotic
(Annexin-V+ /P.I. +)

Figure 9


Neutrophil migration (fold of increase)
18
16
14
12
10
8
6
4
2
0
PBS
IL-8 (25 nM)
fMLP (µM)
Freshly isolated neutrophils
24 hours post-isolation PBS-treated neutrophils
24 hours post-isolation Ang1-treated neutrophils


Neutrophil cellular status (%)
70
60
50
40
30
20
10
0
***
*
***
PBS + + +
PAF (μM) - .001 .01 .1 1 10 - .001 .01 .1 1 10 - .001 .01 .1 1 10
Viable (Annexin V-)
Apoptotic (Annexin V+/P.I.-)
Necrotic (Annexin V+/P.I.+)


Neutrophil cellular status (%)
70
60
50
40
30
20
10
0
***
***
*
PBS
+
+
+
IL-8 (nM)
-
5
10
25
100
200
Viable
(Annexin-V-)
Apoptotic
(Annexin-V+ /P.I. -)
Necrotic
(Annexin-V+ /P.I. +)

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3.0 DISCUSSION

Au cours de mon projet de maîtrise nous avons démontré qu'Ang1 prolonge la viabilité des neutrophiles en retardant leur entrée en phase apoptotique et nécrotique, alors qu'Ang2 ne modulent pas la survie basale des neutrophiles. Nos travaux sont les premiers à démontrer une activité biologique différentielle entre Ang1 et Ang2 sur les neutrophiles. Précedemment, notre laboratoire et d'autres groupes de recherche avons démontré que les deux angiopoïétines avaient des capacités similaires à promouvoir différentes activités pro-inflammatoires sur les neutrophiles, telles que l'adhésion aux CEs, l'activation du CD11b/CD18, la migration et la synthèse du PAF [107, 111, 112]. Bien que l'hypothèse de notre étude se basait sur la capacité des angiopoïétines à moduler la viabilité des neutrophiles via leur capacité à induire la synthèse de PAF, un agent anti-apoptotique connu [192], nous avons démontré que la capacité de synthèse du PAF induite par les angiopoïétines était insuffisante pour permettre l'effet de survie médié par Ang1. De plus, nous avons démontré que la combinaison d'Ang1 avec d'autres médiateurs pro-inflammatoires, soient le PAF et l'IL-8, induit un effet additif sur la viabilité des neutrophiles. Cette observation *in vitro* suggère donc que dans un environnement pro-inflammatoire, plusieurs médiateurs pourraient coopérer à prolonger significativement la survie des neutrophiles et ainsi induire une intensification de l'état inflammatoire. Finalement, nous avons démontré que l'effet d'Ang1 sur la viabilité des neutrophiles est dépendante de la synthèse et de la relâche de l'IL-8, ce qui suggère que l'activité différentielle entre Ang1 et Ang2 sur la viabilité des neutrophiles soit causée par la relâche d'IL-8 puisque notre

laboratoire a démontré que seulement Ang1 induit la relâche de ce médiateur (Neagoe *et coll.,* manuscript sous révision, *J. Cell. Physiol.,* 2011).

3.1 Prémisses et résultats de mes travaux de maîtrise

L'hypothèse de mon projet de maîtrise se basait principalement sur deux séries d'observations. Premièrement, il a été démontré que les angiopoïétines modulent la survie des CEs. En effet, Ang1 prolonge la survie endothéliale [103, 104] alors qu'Ang2 module plutôt la survie endothéliale en fonction des conditions expérimentales; Ang2 augmente la survie endothéliale à fortes concentrations [96, 100] et diminue la viabilité des CEs à plus faibles concentrations [101, 105]. Puisque les angiopoïétines modulent la survie endothéliale et qu'ils induisent plusieurs activités pro-inflammatoires dans les neutrophiles [107, 112], Ang1 et Ang2 pourraient avoir un effet sur la viabilité des neutrophiles. Dans une autre série d'observations, nous avons démontré que les deux angiopoïétines induisent la synthèse du PAF dans les neutrophiles [107] et il a été démontré que le PAF a la capacité de prolonger la survie des neutrophiles [192], nous avons donc émis l'hypothèse qu'Ang1 et Ang2 pourraient prolonger la viabilité des neutrophiles. Cependant, les résultats obtenus au cours de cette étude ont permis de démontrer que seulement Ang1 a la capacité de prolonger la survie des neutrophiles. Bien que cette observation puisse sembler conflictuelle avec les observations qui ont permis d'émettre notre hypothèse de recherche, nos résultats montrent plutôt que les angiopoïétines peuvent avoir à la fois des activités semblables et différentielles sur

les neutrophiles et que la synthèse de PAF induite par les angiopoïétines n'est pas suffisante pour permettre de prolonger la survie des neutrophiles. En effet, bien que notre laboratoire a démontré que les deux angiopoïétines avaient des effets pro-inflammatoires similaires sur les neutrophiles [107, 112], plusieurs groupes de recherche ont démontré qu'Ang1 et Ang2 avaient des effets angiogéniques et inflammatoires différentiels sur les cellules endothéliales. Ang2 était d'ailleurs initialement reconnu comme un antagoniste du récepteur Tie2 [63] et il induit des effets opposés à Ang1 sur la perméabilité vasculaire [72, 88-90, 93]. De plus, Ang1 favorise la survie endothéliale alors que certaines études suggèrent qu'Ang2 accélère l'apoptose des CEs [101, 103-105]. Ces observations suggèrent donc que dans un même type cellulaire, Ang1 et Ang2 pourraient à la fois avoir des effets semblables et différents. Pour ce qui est de la synthèse de PAF induite par les angiopoïétines dans les neutrophiles, elle est environ 4 à 7 fois supérieure à la synthèse de PAF basale [107]. Puisque la quantité de PAF produite par les angiopoïétines est faible et que la concentration optimale de PAF exogène pour prolonger la survie des neutrophiles est de l'ordre de 100 nM [192], il semble que la quantité de PAF produite par Ang1 et Ang2 soit insuffisante pour induire une augmentation de la survie des neutrophiles. De plus, le PAF possède une demie-vie très courte (quelques minutes) [228] et la synthèse de PAF induite par Ang1 et Ang2 se produit très rapidement, soit 7,5 minutes après le traitement avec les angiopoïétines [107]. Contrairement au PAF endogène, le PAF exogène utilisé dans nos expériences pour augmenter la viabilité des neutrophiles est le méthylcarbamyl-PAF C-16, qui est caractérisé par une demie-vie plus longue pour ainsi assurer sa

stabilité et sa capacité d'induire des effets biologiques *in vitro* à long terme. Ainsi, le PAF endogène produit par Ang1 et Ang2 semble donc se dégrader trop rapidement pour permettre d'induire un effet à long terme sur les neutrophiles comme la prolongation de la viabilité qui est généralement observée après 24 heures de traitement.

Après avoir démontré la capacité d'Ang1 à prolonger la viabilité des neutrophiles, nous avons émis l'hypothèse que la combinaison d'Ang1 avec d'autres molécules pro-inflammatoires anti-apoptotiques pourraient avoir un effet additif ou synergique sur la viabilité des neutrophiles. En effet, il a été démontré que les angiopoïétines pouvaient coopérer avec d'autres molécules pro-inflammatoires pour potentialiser leurs effets [109, 110, 112]. Nous avons donc évalué l'effet de deux molécules clés de l'inflammation sur la viabilité des neutrophiles, le PAF et l'IL-8, en combinaison avec Ang1.

Le PAF est un lipide bioactif essentiel à plusieurs processus physiologiques inflammatoires, immunitaires et hémostatiques [228]. Cependant, il est aussi impliqué dans plusieurs conditions pathologiques inflammatoires et thrombotiques [229]. En effet, le PAF agit via l'activation d'un récepteur couplé aux protéines G (RCPG), le PAFR, pour induire l'expression de gènes et la relâche de médiateurs pro-inflammatoires, en plus d'activer directement diverses cellules sanguines, soient les plaquettes, les neutrophiles et les monocytes [230]. De plus, le PAF

favorise l'adhésion des leucocytes et des plaquettes à l'endothélium et augmente la perméabilité vasculaire [231-233]. Le PAF est produit par le métabolisme de l'acide arachidonique via l'activation des phospholipases A_2 dans plusieurs types cellulaires, notamment les CEs et les neutrophiles [106, 234]. Le PAF étant un médiateur inflammatoire important, plusieurs groupes de recherche ont tenté de bloquer son effet par l'utilisation d'antagonistes du PAFR [235] ou d'enzymes recombinantes qui hydrolyse le PAF [236] pour tenter de traiter certaines maladies inflammatoires systémiques, particulièrement le choc septique. Cependant, l'effet de ces traitements n'est pas aussi bénéfique qu'espéré [235-237] puisqu'une forte interaction entre les différents médiateurs inflammatoires existe et il a été suggéré que l'inhibition simultannée de plusieurs molécules inflammatoires, telles que l'IL-8 et le LTB_4, pourrait avoir un effet bénéfique sur les pathologies inflammatoires systémiques [238]. Puisque le PAF interagit avec plusieurs médiateurs et que les angiopoïétines induisent la synthèse du PAF dans les neutrophiles [107], nous avons donc voulu déterminer si la combinaison du PAF et d'Ang1 aurait un effet différent sur la viabilité des neutrophiles par rapport au traitement avec PAF ou Ang1 seuls. Nous avons ainsi démontré que la combinaison du PAF et d'Ang1 produit un effet additif sur la viabilité des neutrophiles et nous avons émis l'hypothèse que la combinaison d'IL-8 et d'Ang1 pourrait également produire un effet additif ou encore synergique sur la viabilité des neutrophiles.

L'IL-8 est un médiateur inflammatoire qui appartient à la famille des chimiokines et qui agit via l'activation de deux récepteurs aux fonctions semblables de type

RCPG, CXCR1 et CXCR2 [239, 240]. Cette puissante molécule est exprimée principalement par les monocytes ainsi que par les macrophages et elle agit sur les monocytes et neutrophiles où elle induit leur activation, la synthèse de lipides inflammatoires (dont le PAF) et leur migration vers le milieu inflammatoire [241-244]. En plus de son rôle inflammatoire, l'IL-8 possède également des effets angiogéniques, ce qui explique son rôle dans une multitude de pathologies autant inflammatoires que vasculaires, dont le syndrome de détresse respiratoire aigu (SDRA), les lésions pulmonaires aiguës, l'athérosclérose ainsi que la croissance tumorale et métastatique [245-249]. L'IL-8 est donc une cible thérapeutique intéressante pour plusieurs pathologies et des traitements à l'aide d'anticorps dirigés contre l'IL-8 ou ses récepteurs ont permis d'inhiber significativement la prolifération tumorale et la formation de métastases chez les patients atteints de mélanomes [250-252]. Cependant, plusieurs chimiokines pourraient coopérer pour supporter la croissance tumorale et il a été proposé que l'inhibition simultanée de plusieurs molécules pourrait amener un effet bénéfique additif [253]. Puisque notre laboratoire a démontré que les angiopoïétines potentialisent l'effet de l'IL-8 sur la migration [112] et que l'IL-8 est un agent anti-apoptotique des neutrophiles [186], nous avons émis l'hypothèse que la combinaison d'Ang1 et d'IL-8 pourrait avoir un effet additif ou synergique sur la viabilité des neutrophiles. Nous avons donc constaté que la combinaison de l'IL-8 et d'Ang1 produit un effet additif sur la viabilité des neutrophiles.

Finalement, nous avons voulu déterminer les médiateurs responsables de l'effet d'Ang1 sur la survie des neutrophiles. Nous avons tout d'abord investigué le rôle du PAF dans l'effet d'Ang1 puisque notre hypothèse de départ se basait sur ce médiateur. L'utilisation d'un antagoniste spécifique du récepteur du PAF, le BN 52021, a permis d'abolir complètement l'effet anti-apoptotique du PAF sur la survie des neutrophiles alors qu'il n'a eu aucun effet sur l'activité anti-apoptotique d'Ang1. Le PAF n'étant pas responsable de l'effet anti-apoptotique d'Ang1 sur les neutrophiles, nous avons par la suite voulu déterminer si l'IL-8 pourrait être impliquée dans l'activité d'Ang1. En effet, au cours de notre étude, notre laboratoire a observé qu'Ang1, à une concentration «physiologique» de 10 nM, induit la synthèse et la relâche d'IL-8 dans les neutrophiles, alors qu'Ang2 n'a aucun effet sur cette activité (Neagoe *et coll.*, manuscript sous révision, *J. Cell. Physiol.,* 2011). De plus, l'effet d'Ang1 sur la relâche d'IL-8 se produit sur une longue période de temps et l'effet est optimal après 6 heures de traitement. Puisque l'effet d'Ang1 sur la viabilité des neutrophiles est optimal à une concentratrion de 10nM et qu'il se produit sur une longue période de temps (effet optimal à 24 heures), alors qu'Ang2 ne modulent pas la survie des neutrophiles, nous avons émis l'hypothèse que la relâche d'IL-8 pourrait être responsable de l'effet d'Ang1. Nous avons donc prétraité des neutrophiles à l'aide d'anticorps bloquants dirigés contre l'IL-8 pour ensuite traiter les neutrophiles avec IL-8 ou Ang1. Nous avons ainsi démontré que l'utilisation d'anticorps bloquants dirigés contre l'IL-8 a permis de supprimer l'effet de l'IL-8 sur la viabilité des neutrophiles et d'abolir presque

complètement l'activité anti-apoptotique d'Ang1 sur les neutrophiles (92 et 80% d'inhibition, respectivement).

Bien que nous avons démontré que l'effet d'Ang1 sur la viabilité des neutrophiles est majoritairement dépendant de la relâche d'IL-8, d'autres médiateurs pourraient être impliqués dans l'activité d'Ang1 puisque les anticorps bloquants dirigés contre l'IL-8 n'ont pas complètement supprimé l'effet d'Ang1. Ainsi, nos études futures devront se concentrer à identifier ces médiateurs, ainsi que les voies de signalisation qui sont impliquées dans l'activité anti-apoptotique d'Ang1 sur les neutrophiles. De plus, l'utilisation de modèles murins *in vivo* d'*air pouch* (modèle inflammatoire) et de *polyvinyl alcohol (PVA) sponge implants* (modèle angiogénique) pourrait nous permettre de mieux comprendre l'impact des résultats de ce projet sur un milieu pro-inflammatoire qui contient une multitude de types cellulaires et de médiateurs, particulièrement pour des pathologies vasculaires inflammatoires où Ang1 est surexprimée.

4.0 CONCLUSION

Nos travaux démontrent pour la première fois qu'Ang1 prolonge la survie des neutrophiles alors qu'Ang2 n'a aucun effet sur cette activité. De plus, nous avons démontré qu'Ang1 peut coopérer avec d'autres médiateurs pro-inflammatoires, soient le PAF et l'IL-8, pour produire un effet additif sur la viabilité des neutrophiles. Finalement, nous avons démontré que l'effet d'Ang1 sur la survie des neutrophiles est dépendant de la relâche d'IL-8 et indépendant de la synthèse de PAF.

Au cours des dernières années, notre laboratoire à démontré que les angiopoïétines avaient un potentiel inflammatoire important, autant au niveau des cellules endothéliales qu'au niveau des neutrophiles [106-108, 112]. Bien que l'implication physiologique et pathologique de ces découvertes soit encore mal comprises, il est évident que les effets angiogéniques et inflammatoires des angiopoïétines peuvent coopérer pour faciliter l'établissement d'une angiogenèse pathologique, qui est caractérisée par un environnement pro-inflammatoire. Plusieurs pathologies sont d'ailleurs caractérisées par une sur-expression des angiopoïétines, comme le cancer tumoral et l'arthrite rhumatoïde [114, 254]. Il est donc essentiel d'identifier le rôle exact et les mécanismes des angiopoïétines dans ces pathologies afin d'identifier des cibles thérapeutiques potentielles. Nos études futures se concentreront donc à augmenter nos connaissances sur le rôle inflammatoire des angiopoïétines dans des modèles murins inflammatoires *in vivo,* tel que le modèle du *air pouch*, et à

identifier les mécanismes responsables de ces activités dans des modèles humains *in vitro*. De plus, nous tenterons d'évaluer les concentrations sériques et tissulaires d'Ang1, d'Ang2 et de Tie2 dans des individus atteints de pathologies inflammatoires vasculaires, telles que la vascularite, la polymyosite et l'arthrose.

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6.0 ANNEXES

ANNEXE 1

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Angiopoietin-1 but not angiopoietin-2 promotes neutrophil viability: Role of interleukin-8 and platelet-activating factor

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ABSTRACT

We previously reported the expression of angiopoietin receptor Tie2 on human neutrophils. Both angiopoietins (Ang1 and Ang2) induce platelet activating factor (PAF) synthesis from endothelial cells (ECs) and neutrophils. Both angiopoietins can also modulate EC viability and since PAF can promote pro-survival activity on neutrophils, we addressed whether Ang1 and/or Ang2 could modulate neutrophil viability. Neutrophils were isolated from venous blood of healthy volunteers and neutrophil viability was assessed by flow cytometry using apoptotic and necrotic markers (annexin-V and propidium iodide (P.I.), respectively). Basal neutrophil viability from 0 to 24 h post-isolation decreased from 98% to ≈45%. Treatment with anti-apoptotic mediators such as interleukin-8 (IL-8; 25 nM) and PAF (100 nM) increased neutrophil basal viability by 34 and 26% (raising it from 43 to 58 and 55%) respectively. Treatment with Ang1 (0.001–50 nM) increased neutrophil viability by up to 41%, while Ang2 had no significant effect. Combination of IL-8 (25 nM) or PAF (100 nM) with Ang1 (10 nM) further increased neutrophil viability by 56 and 60% respectively. We also observed that Ang1, but not Ang2 can promote IL-8 release and that a pretreatment of the neutrophils with blocking anti-IL-8 antibodies inhibited the anti-apoptotic effect of IL-8 and Ang1 by 92 and 81% respectively. Pretreatment with a selective PAF receptor antagonist (BN 52021), did abrogate PAF pro-survival activity, without affecting Ang1-induced neutrophil viability. Our data are the first ones to report Ang1 pro-survival activity on neutrophils, which is mainly driven through IL-8 release.

1. Introduction

Angiopoietins (Ang1 and Ang2) are growth factors structurally related, and contributing to angiogenesis upon binding to Tie2 receptor [1]. Ang1 is known as a Tie2 agonist capable of inducing EC migration and proliferation [2–5]. In addition, Ang1 promotes pericyte recruitment to the newly formed vessels, therefore leading to the maturation and stabilization of the vasculature [6,7]. Furthermore, Ang1 has the capacity to reduce apoptosis of many cellular types such as EC, cardiomyocytes and mesenchymal stem cells [8–11]. This activity can lead to a protective role of Ang1 in acute inflammatory conditions such as in lung injury, and in chronic diseases such as cardiac remodeling where Ang1 improves cardiomyocytes survival and reduces cardiac hypertrophy [12,13]. In contrast, Ang2 was initially reported as a Tie2 antagonist, inducing vascular permeability and EC apoptosis, therefore, leading to the promotion of a pro-inflammatory environment and the destabilization of pre-existing vasculature [14,15]. However, mounting evidence demonstrates that Ang2 could also have agonistic properties by being able to induce Tie2 phosphorylation, EC chemotaxis and *in vitro* tubule capillary-like formation [16–19]. In previous studies, we reported that both angiopoietins exert pro-inflammatory activities on ECs by their capacity to stimulate PAF synthesis and P-selectin translocation, therefore leading to neutrophil adhesion onto activated ECs and transmigration [20–22]. Furthermore, angiopoietins can also modulate cytokine activities in ECs, for instance, at low concentrations, Ang2 is a priming factor for tumor necrosis factor-α (TNF-α) [23] and both Ang1 and Ang2 have been reported to potentiate VEGF-induced angiogenesis in a mouse cornea model [1].

We also reported the expression of Tie2 receptor on human neutrophils, and that both angiopoietins can promote pro-inflammatory activities on neutrophils [22]. Ang1 and Ang2 support neutrophil PAF synthesis, activation of CD11b/CD18 (β_2-integrin), adhesion and migration [22,24]. In addition, angiopoietins can also enhance the activities of pro-inflammatory mediators, for instance, pretreatment with Ang1 and/or Ang2 potentiates IL-8-induced neutrophil migration [24].

Neutrophil lifespan is relatively short, ranging from 8 to 20 h in blood circulation and up to few days in perivascular tissues [25–27]. Neutrophils are the primary leukocytes being recruited at inflammatory sites and promote a range of pro-inflammatory activities, namely by

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their capacity to release various chemokines and cytokines, including VEGF, TNF-α, IL-1, IL-8 and PAF [28,29]. Neutrophils can also secrete catalytic enzymes (metalloproteinases, lysosymes), which contribute to extracellular matrix degradation and elimination of pathogens [29,30]. Increasing neutrophil viability can prolong and enhance the inflammatory response, thus, contributing to the development and progression of multiple vascular-associated diseases such as systemic inflammatory response syndrome (SIRS), tumor growth, ischemia/reperfusion injury and rheumatoid arthritis (RA) [26,31–35].

For instance, it has been reported that the expression of both angiopoietins and members of Tie-receptor family (Tie1 and Tie2) is increased in patients suffering from RA, and that the corresponding expression is not limited to vascular cells, but also overexpressed in stromal cells and in the synovial lining [36]. Such overexpression could lead to the development of pathological angiogenesis, which can be inhibited by the blockade of Ang/Tie ligand–receptor system [37]. In addition, since RA is characterized as a chronic inflammatory disease, angiopoietins could contribute to the inflammatory process through their capacity to promote neutrophil-mediated pro-inflammatory responses, namely through PAF and IL-8 participation [22,24,38,39].

PAF is a bioactive lipid that derivates from arachidonic acid metabolism acting through the stimulation of a G-protein-coupled receptor (PAFR). This potent pro-inflammatory mediator is involved in inflammation, reproduction, angiogenesis and tumor growth metastasis [40]. PAF activates and is produced by many inflammatory cells including ECs, platelets, monocytes, lymphocytes and neutrophils. In addition, in function of the cell type, PAF promotes many pro-inflammatory activities such as platelet aggregation and modulation of cell viability [41]. For instance, PAF promotes apoptosis on hepatocytes and neurons, whereas it can prolong cell viability of various leukocytes, including neutrophils [40,42].

IL-8 was first described as a monocyte-derived neutrophil chemotactic factor (MDNCF) [43–45], and subsequently reported for being synthesized by ECs, fibroblasts and keratinocytes [46,47]. IL-8 is a major pro-inflammatory mediator, acting predominantly on neutrophils where it induces respiratory burst [48], generation of superoxide and hydrogen peroxide [49], cellular reorganization [49], secretion of catalytic enzymes (metalloproteinases, lysosymes) [50], synthesis of bioactive lipids [51], translocation and activation of adhesion molecules [52], chemotaxis [53] and pro-survival activity [54]. IL-8 is also involved in the development of various pathological conditions, such as acute respiratory distress syndrome (ARDS), ischemia/reperfusion injury, tumor growth and metastasis [46,54].

Since both angiopoietins can modulate endothelial cell survival [10,11,17,55–57] and induce PAF synthesis [21,22] which supports neutrophil viability [42], and that both angiopoietins can modulate IL-8 chemotactic activity on neutrophils [24], we thus wanted to assess whether Ang1 and/or Ang2 can modulate neutrophil viability, and if so, delineate the potential contribution of specific cytokines being synthesized and released by the neutrophils.

2. Material and methods

2.1. Neutrophil isolation and purification

Human venous blood was obtained from consent healthy donors free from medication for at least 10 days prior to the experiments and in accordance with the guidelines of the Montreal Heart Institute's ethical committee. Neutrophils were isolated as described previously [22,24,58]. Neutrophils were then resuspended in RPMI medium (Lonza, Basel, Switzerland) supplemented with 25 mM Hepes (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) and 1% penicillin/streptomycin. At least 95% of isolated cells were neutrophils as determined by staining with Kwik Diff solutions according to the manufacturer's instructions (Thermo Fisher Scientific, Kalamazoo, MI, USA) and viability was found to be greater than 98% as assessed by Trypan blue dye exclusion assay [59].

2.2. Assessment of viability, apoptosis and necrosis

Neutrophils were incubated in 24-well plates (Becton Dickinson, San Jose, CA, USA) at 5×10^6 cells/well for different periods of time (0–24 h) at 37 °C and treated with various agonists: Ang1 and Ang2 (R&D Systems, Minneapolis, MN, USA), IL-8 (Peprotech, Rocky Hill, NJ, USA) and methylcarbamyl-PAF C-16 (PAF; Cayman Chemical, Ann Arbor, MI, USA) in RPMI medium supplemented with 10% human serum (Millipore, Billerica, MA, USA). In some experiments, neutrophils were preincubated with a secreted phospholipase A_2 ($sPLA_2$) inhibitor, 12-epi-scalaradial (Biomol, Plymouth Meeting, PA, USA), a cytosolic PLA_2 ($cPLA_2$) inhibitor, $AACOCF_3$ (Cayman Chemical), a PAF receptor antagonist, BN 52021 (Sigma-Aldrich, St. Louis, MO, USA), a goat polyclonal blocking anti-human Tie2 antibody (R&D Systems, #AF313), a goat polyclonal blocking anti-human IL-8 antibody (R&D Systems, #AB-208-NA) or control IgG isotype for 30 min to 2 h prior to the addition of selected agonists. Viability was measured by flow cytometry using annexin-V-fluorescein isothiocyanate (FITC) and P.I. (Becton Dickinson) as previously described [60,61]. Briefly, neutrophils (1×10^6 cells/50 μl) were rinsed with PBS, centrifuged (900 *g*, 7 min at 4 °C), resuspended in annexin-V binding buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) (Becton Dickinson) and incubated in the dark for 15 min at room temperature in the presence of 2.5 μl of annexin-V-FITC (20 μg/ml in aqueous buffered solution containing BSA and 0.09% sodium azide) and 5 μl of P.I. (50 μg/ml in PBS, pH 7.4) (PharMingen, Becton Dickinson). Flow cytometry analyses (15 000 events per tube) were performed using a flow cytometer with a 488 nm wavelength argon laser (Coulter EPICS XL, Beckman Coulter, Brea, CA, USA). Intact neutrophils were selected according to size and granularity. Cells negative for both annexin-V and P.I. were considered as viable (annexin-V^-/P.I.$^-$), annexin-V-positive and P.I.-negative cells were considered as early apoptotic (annexin-V^+/P.I.$^-$) and cells positive for both markers were considered as necrotic and/or in late apoptotic phase (annexin-V^+/P.I.$^+$).

2.3. Morphological assessment of neutrophil apoptosis

Viability was also assessed by morphological observations as previously described [27]. Neutrophils (5×10^5 cells/condition) were cytocentrifuged, fixed and stained for 30 s with hematoxylin QS (Vector Laboratories, Burlingame, CA, USA). Neutrophils were examined under oil immersion light microscopy at 400× magnification. Cells in five random fields per condition were analyzed and apoptotic/necrotic cells were characterized by condensed and darkly stained nuclei. At least 400 neutrophils were counted per condition. Flow cytometry and morphological assessment gave similar results in regard to neutrophil viability.

2.4. Neutrophil chemotactic activity

In vitro chemotactic assays were performed in Transwell-96 well permeable supports. The top and the bottom well were separated by a porous polycarbonate membrane filter (3 μm pore size) (Corning Life Science, Corning, NY) as previously described [62,63]. Briefly, the bottom wells were loaded with RPMI containing PBS, IL-8 (25 nM) or fMLP (0.1–10 μM) to a final volume of 230 μl. The top wells were loaded with neutrophils (10^7 cells/ml; 80 μl from a RPMI suspension). In function of the experiments, neutrophils were freshly added post-isolation or pretreated for 24 h with PBS or Ang1 (10 nM) prior to the chemotactic assay. The Transwell-96 well permeable supports were incubated at 37 °C for 1 h in a humidified incubator in the presence of 5% CO_2. At the end of the incubation period, the supernatant from

the bottom wells was collected, and a count of migrating neutrophils was assessed with an Amnis ImageStream system.

2.5. Statistical analysis

Results from each experiment are the mean of cumulative independent experiments from the blood of at least three independent donors, and expressed as the mean ± SEM. Comparisons were made by analysis of variance (ANOVA) followed by a Dunnett's test. Differences were considered significant at p values ≤ 0.05.

3. Results

3.1. Ang1, but not Ang2, promotes neutrophil viability

It has been reported that Ang1 and Ang2 modulate EC survival while PAF increases neutrophil viability [10,11,17,42,56,57,64]. On our side, we have reported that both angiopoietins induce PAF synthesis in neutrophils [22]. Thus, we sought to determine whether angiopoietins could modulate neutrophil viability and the potential role of PAF. In a first series of experiments we assessed the survival rate of neutrophils on a 24-hour period. Upon neutrophil isolation, the viability of the cells was ≈98% and declined time-dependently to ≈45% after 24 h. We then used PAF as a positive control to promote neutrophil viability. Upon a 24-hour incubation period, we observed a bell-shape concentration-dependent anti-apoptotic effect of PAF (1–10 000 nM) on neutrophil viability with an optimal effect being achieved at 100 nM, providing a 26% increase of neutrophil viability (raising the basal viability from 43.4 to 54.8%) (Suppl. data Fig. 1).

We then assessed the capacity of both angiopoietins (Ang1 and Ang2) to modulate neutrophil viability on a concentration- and time-dependent manner. Treatment with Ang1 (0.001–50 nM; 0.07–3500 ng/ml) increased significantly neutrophil viability at 1, 10 and 50 nM, raising the basal viability from 43.4 to 53.3, 59.0 and 61.3% respectively (corresponding to a 23, 36 and 41% increase). Interestingly, in the current set of experiments, Ang1 at 10 nM was already more potent than PAF (100 nM) to promote neutrophil viability (Fig. 1A). To assess whether Ang1-mediated pro-survival activity was caused by an effect on apoptosis or necrosis, we evaluated the effect of Ang1 on neutrophil viability with two specific markers of membrane asymmetry, annexin-V-FITC (phosphatidylserine externalization; apoptosis) and P.I. (intercalating agent; necrosis). These data were further confirmed by cytologic staining and morphological assessment of neutrophil viability under optical microscopy. The beneficial effect of Ang1 on neutrophil viability is observed by its capacity to reduce the apoptotic and necrotic events. In fact, PAF and Ang1 had a similar capacity to significantly lower neutrophil apoptosis (reducing it from 38.9% to 34.1% and 29.4% respectively). In addition, PAF and Ang1 had a similar capacity to significantly lower neutrophil necrosis (reducing it from 15.9% to 8.8% and 9.7% respectively) (Fig. 1A). In parallel, we performed the same experiments with neutrophils from the same donors and observed that Ang2 (0.001–50 nM; 0.066–3300 ng/ml) as opposed to Ang1, had no significant effect on neutrophil viability (Fig. 1B). As the maximal effect mediated by Ang1 was nearly achieved at 10 nM, we elected to limit the use of Ang1 up to 10 nM for upcoming experiments.

We then treated the neutrophils with the angiopoietins (Ang1, Ang2; 1 and 10 nM) and PAF (100 nM; positive control) on a 24-hour time period to address the kinetic of Ang1-mediated anti-apoptotic activity. Treatment with Ang1 (1 and 10 nM; 70 and 700 ng/ml) provided a positive trend on neutrophil viability upon 12 h post-isolation, and the Ang1 anti-apoptotic effect was significant and maximal (10 nM) at 24 h (Fig. 2A). By performing a time-response kinetic, we were led to observe that Ang1 (1 and 10 nM) had a beneficial pro-survival activity by delaying the entry of neutrophils into apoptotic and necrotic phases (Fig. 2B–C). Treatment with PAF (100 nM) or Ang1 (1 and 10 nM) at 24 h significantly reduced neutrophil apoptosis by 12, 13 and 24%

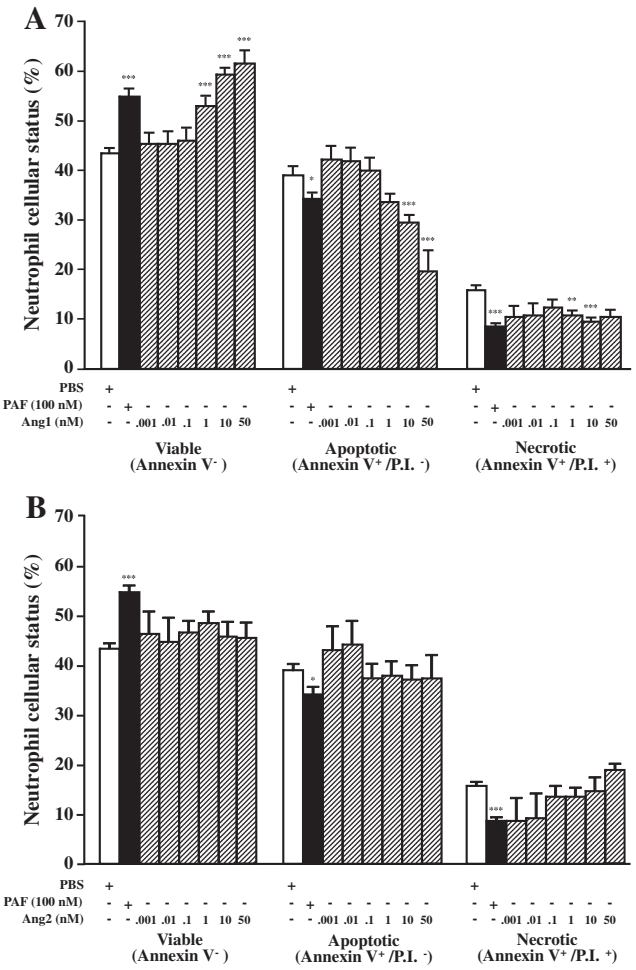

Fig. 1. Effect of angiopoietins on neutrophil viability in a concentration-dependent manner. Human neutrophils were incubated with PAF (100 nM; positive control), Ang1 (0.001–50 nM) (A) and Ang2 (0.001–50 nM) (B) for 24 h at 37 °C. Neutrophils were considered viable in the absence of positive staining for both markers (annexin-V and P.I.; annexin-V^-/P.$I.^-$). Neutrophils were considered under apoptotic state when they stained positively for annexin-V and negatively for P.I. (annexin-V^+/P.$I.^-$). Neutrophils were considered necrotic when they stained positively for both markers (annexin-V^+/P.$I.^+$). *p<0.05; **p<0.01 and ***p<0.001 as compared to PBS-treated cells.

respectively. In addition, treatment with PAF (100 nM) or Ang1 (1 and 10 nM) at 24 h also significantly decreased neutrophil necrosis by 45, 31 and 39% respectively (Fig. 2C). Since pro-survival activity of Ang1 and PAF on neutrophils was optimal at 24 h, our experiments were subsequently performed at 24 h post-isolation. The same experiments were performed in parallel with Ang2 (1 and 10 nM; 66 and 660 ng/ml). Again, we observed that Ang2 had no effect on neutrophil viability, apoptosis and necrosis from 0 to 24 h post-isolation (Fig. 2A–C).

3.2. Ang1-mediated pro-survival activity is Tie2-dependent

In previous studies, we and other groups observed that angiopoietins could mediate their biological activities upon binding and activation of Tie2 receptor [22,24,65,66]. Thus, we wanted to assess if the pro-survival activity of Ang1 is implying Tie2 participation. In the current set of experiments, neutrophils were pretreated with a goat polyclonal blocking anti-human Tie2 antibody (5, 10 or 20 μg/ml) or control goat IgG isotype (20 μg/ml) for 1 h before stimulation with PBS or Ang1 (10 nM) for an additional 23 h. Treatment with control goat IgG or with anti-Tie2 blocking antibodies (20 μg/ml) had no significant effect on basal neutrophil viability (Fig. 3). Pretreatment of

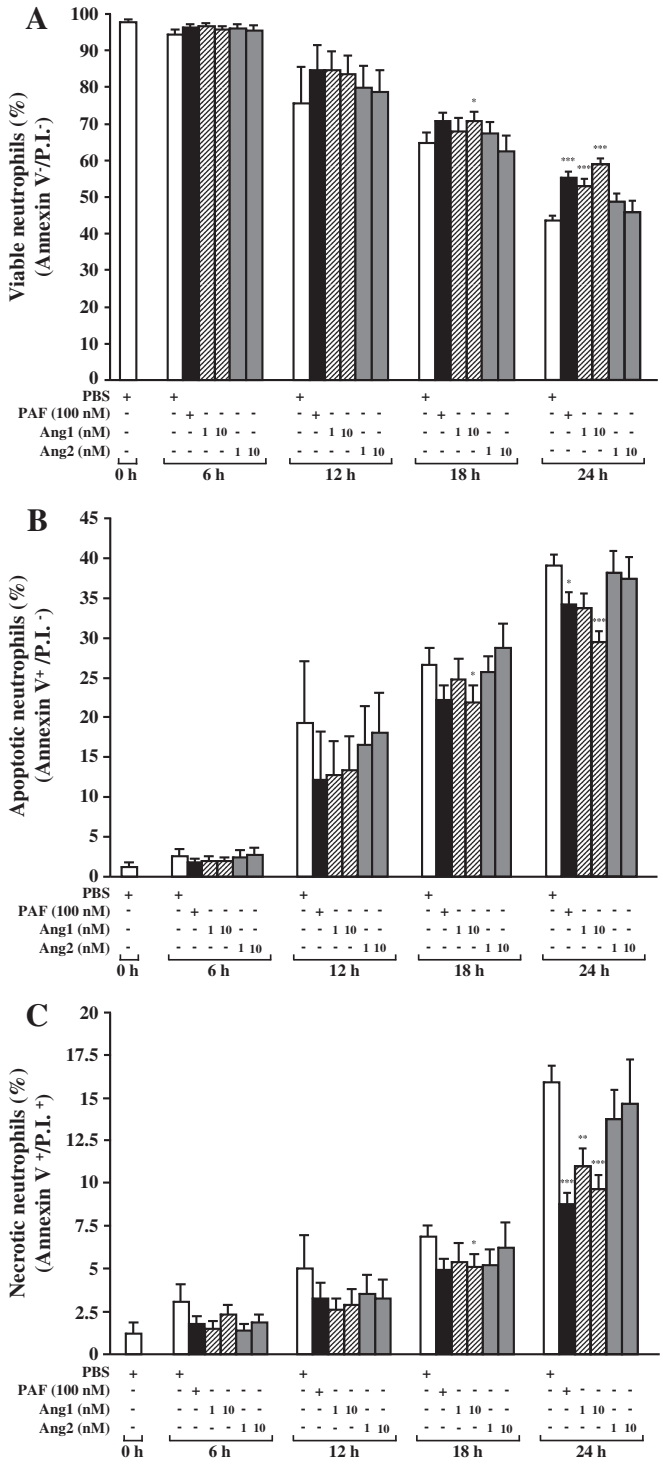

Fig. 2. Effect of Ang1 and Ang2 on neutrophil viability in a time-dependent manner. Neutrophils were incubated with Ang1 (1–10 nM), Ang2 (1–10 nM) and PAF (100 nM) from 0 to 24 h at 37 °C. (A) Viable neutrophils, (B) apoptotic neutrophils and (C) necrotic neutrophils. *p<0.05; **p<0.01 and ***p<0.001 as compared to PBS-treated cells.

neutrophils with anti-Tie2 blocking antibodies reduced in a concentration-dependent manner the pro-survival activity of Ang1 with a maximal effect being achieved at 20 μg/ml (84% inhibition) (Fig. 3). Pretreatment of neutrophils with anti-Tie2 blocking antibodies did not reverse Ang1 mediated anti-apoptotic activity but did abrogate Ang1-anti-necrotic activity (Fig. 3).

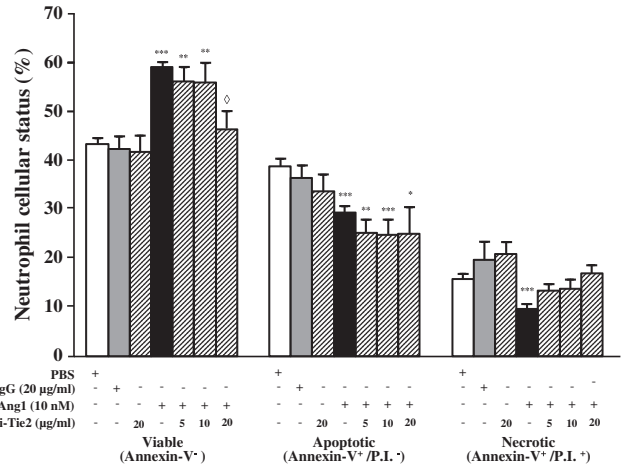

Fig. 3. Blocking anti-Tie2 antibodies reduce Ang1-mediated pro-survival effect on neutrophils. Neutrophils were pretreated with or without blocking anti-human Tie-2 IgG antibodies (5–20 μg/ml) for 1 h. Then, neutrophils were incubated with or without Ang1 (10 nM) for 23 h at 37 °C. *p<0.05; **p<0.01; ***p<0.001 as compared to PBS-treated cells; ◊p<0.05 as compared to Ang1-treated cells (10 nM).

3.3. Additive effect of PAF and Ang1 on neutrophil viability

Since PAF is a neutrophil pro-survival mediator [42], and as observed in our current study, we thus wanted to address whether both angiopoietins and PAF could cooperate on neutrophil viability. Neutrophils were treated with Ang1 (0.1–10 nM) in the presence or absence of PAF (100 nM) for 24 h. Treatment with PAF or Ang1 (1 and 10 nM) increased neutrophil viability by 26, 23 and 36% respectively (Fig. 4). Combining PAF (100 nM) with Ang1 (1 and 10 nM) provided an additive effect, increasing by 48 and 60% the viability of the neutrophils (raising the basal neutrophil viability from 43.4 to 64.4 and 69.6%, respectively). Furthermore, the combination of PAF (100 nM) with Ang1 (1 and 10 nM) provided a significant increase of neutrophil survival as compared to the cells treated either with PAF (100 nM) or Ang1 (1 or 10 nM) alone (Fig. 4). Neutrophils were also treated with Ang1 (0.1 nM) to assess whether PAF (100 nM) could prime an effect of Ang1 at a concentration, which by itself does not induce neutrophil viability. This latter combination did not provide an additive effect on neutrophil viability (Fig. 4). In addition, treatment of neutrophils with PAF or Ang1 provided a significant

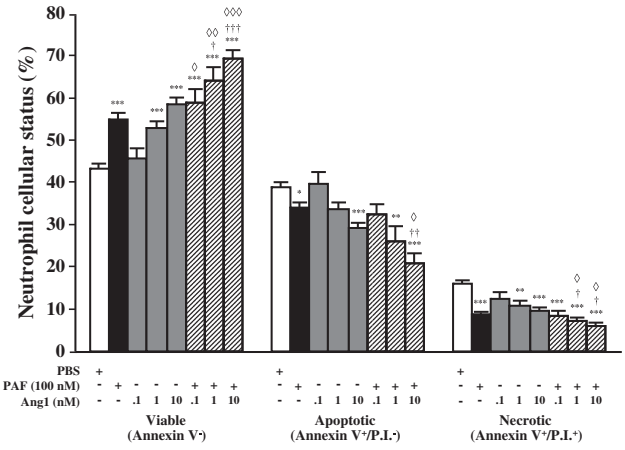

Fig. 4. Combination of PAF and Ang1 provides an additive effect on neutrophil viability. Neutrophils were incubated with Ang1 (0.1–10 nM) in the presence or absence of PAF (100 nM) for 24 h at 37 °C. *p<0.05; **p<0.01; ***p<0.001 as compared to PBS-treated cells; ◊p<0.05; ◊◊p<0.01; ◊◊◊p<0.001 as compared to Ang1-treated cells (0.1, 1 and 10 nM); †p<0.05; ††p<0.01 and †††p<0.001 as compared to PAF-treated cells (100 nM).

decrease of apoptosis and their combination (PAF; 100 nM and Ang1; 10 nM) provided an additive capacity to decrease neutrophil apoptosis as compared to the cells treated either with PAF (100 nM) or Ang1 (10 nM) alone (Fig. 4). Finally, the combination of PAF (100 nM) with Ang1 (1 and 10 nM) provided a significant decrease of neutrophil necrosis as compared to the cells treated either with PAF (100 nM) or Ang1 (1 or 10 nM) alone (Fig. 4).

3.4. Contribution of PAF on Ang1 pro-survival activity

In the current setting of experiments, we wanted to address if endogenous PAF is involved in Ang1 pro-survival activity on neutrophils. We thus pretreated neutrophils with a selective PAF receptor antagonist (BN 52021; 50 and 100 μM) [67,68], or the corresponding control vehicle (DMSO) for 1 h prior to the addition of PAF (100 nM) or Ang1 (10 nM) for an additional 23 h. Treatment with BN 52021 alone did not modulate basal neutrophil viability. In pretreatment, BN 52021 abrogated completely PAF-mediated pro-survival activity, bringing the survival rate of the neutrophil even below to the control-PBS level (Fig. 5). In addition, a pretreatment with BN 52021 abrogated as well the PAF-mediated anti-apoptotic effect on neutrophils (Fig. 5). On the other hand, a pretreatment with BN 52021 did not modulate significantly the effect of Ang1 on neutrophil viability, apoptosis and necrosis (Fig. 5). We previously reported that in ECs, both angiopoietins induce PAF synthesis through the activation of $sPLA_2$-V and independently from $cPLA_2$ activation [21]. In function of the stimulus, both PLA_2s can be activated to promote neutrophil PAF synthesis [69–73]. We thus pretreated the neutrophils with a $sPLA_2$ inhibitor (scalaradial; up to 20 μM) [21,74,75], a $cPLA_2$ inhibitor ($AACOCF_3$; up to 20 μM) [21,74–76] or their corresponding control vehicles (DMSO or ethanol, respectively) for 1 h prior to the addition of Ang1 (10 nM) for an additional 23 h. Treatment with both inhibitors ($AACOCF_3$ and scalaradial) did not modulate Ang1 pro-survival activity on neutrophils (data not shown).

3.5. Additive effect of IL-8 and Ang1 on neutrophil viability

We reported that both angiopoietins can potentiate IL-8-induced neutrophil migration [24] and since IL-8 is a neutrophil pro-survival mediator [54], we wanted to assess if the angiopoietins and IL-8 could cooperate on neutrophil viability. First, we addressed the capacity of IL-8 to promote neutrophil viability. Upon a 24-hour incubation period, we observed a bell-shape concentration-dependent anti-apoptotic effect of IL-8 (5–200 nM) on neutrophil viability with an optimal effect being achieved at 25 nM, providing a 34% increase of neutrophil viability (raising the basal viability from 43.4 to 58.0%) (Suppl. data Fig. 2). Neutrophils were then treated with Ang1 (0.1–10 nM) in the presence or absence of IL-8 (25 nM) for 24 h. The combination of IL-8 (25 nM) with Ang1 (10 nM) provided an additive effect, increasing basal neutrophil viability by 56%. This combination provided also a significant increase of neutrophil viability as compared to cells treated with Ang1 (10 nM) alone (Fig. 6). Neutrophils were also treated with Ang1 (0.1 nM) to assess whether IL-8 (25 nM) could prime an effect of Ang1 at a concentration, which by itself, does not provide a significant effect on neutrophil viability (Fig. 6). In addition, IL-8 or Ang1-treated neutrophils had a significant decrease of cell apoptosis, which was also significant upon the combination of IL-8 (25 nM) and Ang1 (10 nM) (Fig. 6). Finally, treatment of the neutrophils with IL-8 (25 nM) and Ang1 (10 nM), alone or combined, led to a significant decrease of cell necrosis (Fig. 6).

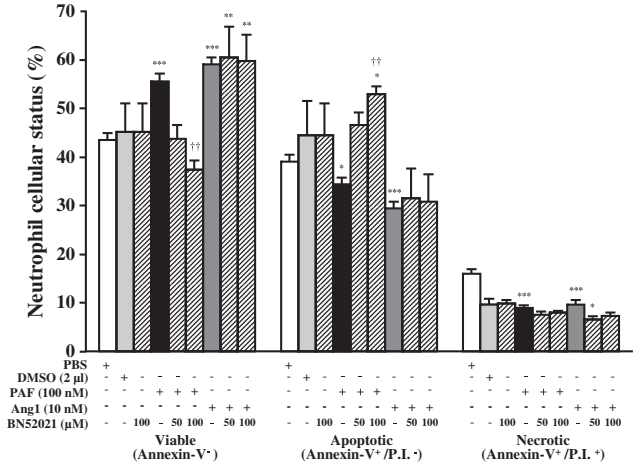

Fig. 5. BN 52021, a PAF receptor antagonist, did not reduce Ang1-mediated pro-survival effect on neutrophils. Neutrophils were pretreated with or without BN 52021 (50 or 100 μM) or with DMSO (2 μl; concentration used to dissolve BN 52021) for 1 h. Then, neutrophils were incubated with or without PAF (100 nM) and/or Ang1 (10 nM) for 24 h at 37 °C. *p<0.05; **p<0.01; ***p<0.001 as compared to PBS-treated cells; ††p<0.01 as compared to PAF-treated cells (100 nM).

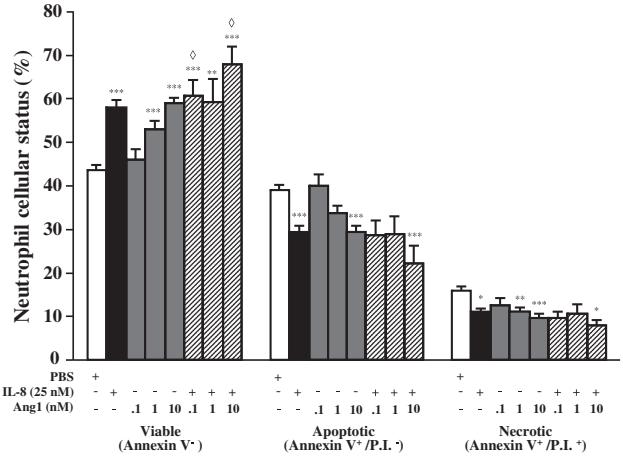

Fig. 6. Combination of IL-8 and Ang1 provides an additive effect on neutrophil viability. Neutrophils were incubated with Ang1 (0.1–10 nM) in the presence or absence of IL-8 (25 nM) for 24 h at 37 °C. *p<0.05; **p<0.01; ***p<0.001 as compared to PBS-treated cells; ◊p<0.05 as compared to Ang1-treated cells (0.1 and 10 nM).

3.6. Contribution of IL-8 on Ang1 pro-survival activity

Both angiopoietins are known to promote similar pro-inflammatory activities in neutrophils [22,24]. However, in our current set of experiments, we demonstrate differential activities of Ang1 and Ang2 on neutrophil viability. In addition, we recently observed that Ang1, but not Ang2, induces IL-8 synthesis and release from neutrophils in a concentration-dependent manner with an optimal effect at 10 nM and within 2 h post-stimulation [77]. Therefore, we wanted to address whether IL-8 was implicated in Ang1-induced neutrophil viability. Neutrophils were pretreated with goat anti-human IL-8 blocking IgG antibodies (20–100 μg/ml) or control goat IgG isotype (100 μg/ml) for 2 h before stimulation with PBS, IL-8 (25 nM) or Ang1 (10 nM) for an additional 22 h. Treatment with control goat IgG or with anti-IL-8 blocking antibodies (100 μg/ml) had no effect on basal neutrophil viability (Fig. 7). Pretreatment with anti-IL-8 blocking antibodies reduced both IL-8 (25 nM) and Ang1 (10 nM)-induced neutrophil viability in a concentration-dependent manner with a maximal effect at 100 μg/ml for IL-8 (92% decrease) and 50 μg/ml for Ang1 (81% decrease) (Fig. 7). In addition, treatment with IL-8 significantly decreased both apoptotic and necrotic neutrophils, whereas pretreatment with anti-IL-8 blocking antibodies (100 μg/ml) provided a trend to reverse the effect of IL-8 on neutrophil apoptosis, and significantly reversed the effect of IL-8 on

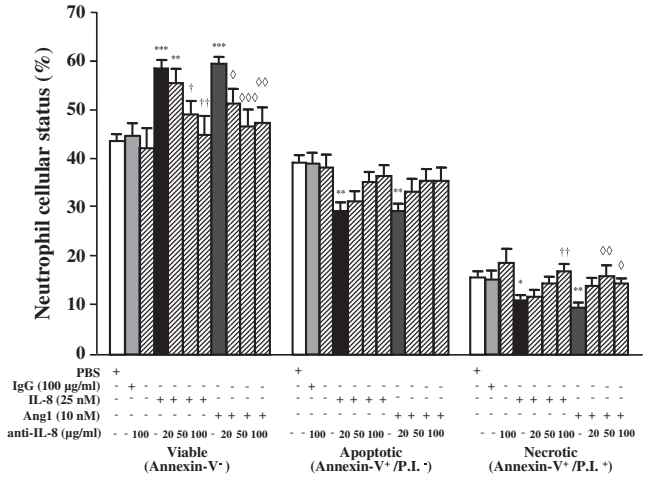

Fig. 7. Blocking anti-IL-8 antibodies reduce Ang1-mediated pro-survival effect on neutrophils. Neutrophils were pretreated with or without blocking anti-human IL-8 IgG antibodies (20–100 μg/ml) for 2 h. Then, neutrophils were incubated with or without IL-8 (25 nM) or Ang1 (10 nM) for 22 h at 37 °C. *$p<0.05$; **$p<0.01$; ***$p<0.001$ as compared to PBS-treated cells; ◊$p<0.05$; ◊◊$p<0.01$; ◊◊◊$p<0.001$ as compared to Ang1-treated cells (10 nM); †$p<0.05$ and ††$p<0.01$ as compared to IL-8-treated cells (25 nM).

neutrophil necrosis. Similarly, Ang1 also decreased significantly neutrophil apoptosis and necrosis, whereas pretreatment with anti-IL-8 blocking antibodies (50 and 100 μg/ml) significantly reversed only the effect of Ang1 on neutrophil necrosis while having a trend to reverse the effect of Ang1 on neutrophil apoptosis (Fig. 7).

3.7. Contribution of IL-8 on PAF pro-survival activity

Since Ang1 and PAF are both capable to promote IL-8 synthesis and release by the neutrophils [77], and that Ang1 pro-survival activity is IL-8-dependent, we thus wanted to address if PAF pro-survival activity is also IL-8-dependent. Neutrophils were pretreated with goat anti-human IL-8 blocking IgG antibodies (20–100 μg/ml) or control goat IgG isotype (100 μg/ml) for 2 h before stimulation with PBS or PAF (100 nM) for an additional 22 h. Treatment with control goat IgG or with anti-IL-8 blocking antibodies (100 μg/ml) had no effect on basal neutrophil viability (Fig. 8). Pretreatment with anti-IL-8 blocking antibodies (20, 50 and 100 μg/ml) reduced significantly PAF-induced neutrophil viability in a concentration-dependent manner with a maximal effect at 100 μg/ml (92% decrease) (Fig. 8). In addition, treatment with PAF significantly decreased both apoptotic and necrotic neutrophils, whereas pretreatment with anti-IL-8 blocking antibodies (20, 50 and 100 μg/ml) provided a trend to reverse the effect of PAF on neutrophil apoptosis, and significantly reversed the effect of PAF on neutrophil necrosis (Fig. 8).

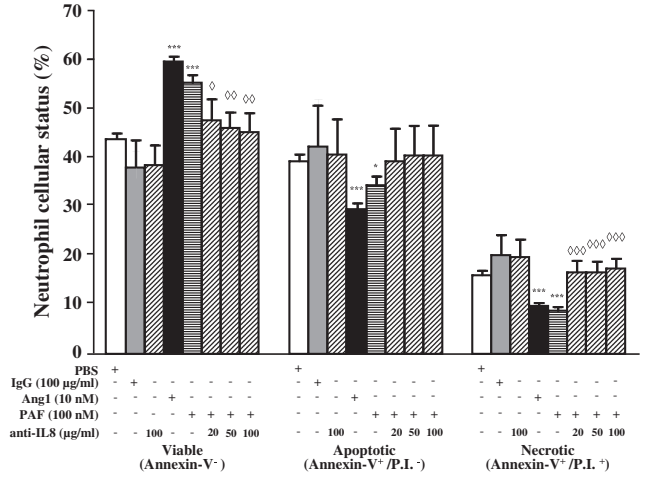

Fig. 8. Blocking anti-IL-8 antibodies reduce PAF-mediated pro-survival effect on neutrophils. Neutrophils were pretreated with or without blocking anti-human IL-8 IgG antibodies (20–100 μg/ml) for 2 h. Then, neutrophils were incubated with or without PAF (100 nM) for 22 h at 37 °C. *$p<0.05$ and ***$p<0.001$ as compared to PBS-treated cells; ◊$p<0.05$; ◊◊$p<0.01$; ◊◊◊$p<0.001$ as compared to PAF-treated cells.

3.8. Neutrophil chemotactic activity

We previously reported the capacity of Ang1 and IL-8 to promote neutrophil migration [24]. Herein, we wanted to ascertain if the neutrophils upon a 24-hour incubation period either under PBS or Ang1 treatment were still capable to migrate upon stimulation by pro-chemotactic mediators such as IL-8 and fMLP. On freshly isolated neutrophils, IL-8 (25 nM) [24] increased by 6.3-fold the migration of neutrophils upon a 60-minute incubation period. Similarly, a treatment with fMLP (0.1–10 μM) [78,79] provided a bell-shaped increase of neutrophil migration with a peak mediated at 1 μM (13.9-fold) (Fig. 9). Upon a 24-hour incubation period in control media containing either control vehicle (PBS) or Ang1 (10 nM), we performed a 60-minute chemotactic assay and observed that the neutrophils maintained a similar capacity to respond to IL-8 (7.8- and 6.5-fold of increase respectively) and fMLP (1 μM; 9.3- and 8.1-fold of increase respectively) (Fig. 9).

4. Discussion

In the present study, we report for the first time that upon Tie2 activation, Ang1 has a pro-survival activity on neutrophils, whereas Ang2 has no effect. The combination of known pro-inflammatory and pro-survival mediators for the neutrophils, namely PAF and IL-8, provided an additive effect on Ang1-mediated pro-survival activity. In addition, using a selective PAF receptor antagonist and blocking IL-8 antibodies, we delineated that Ang1 pro-survival activity is IL-8 dependent.

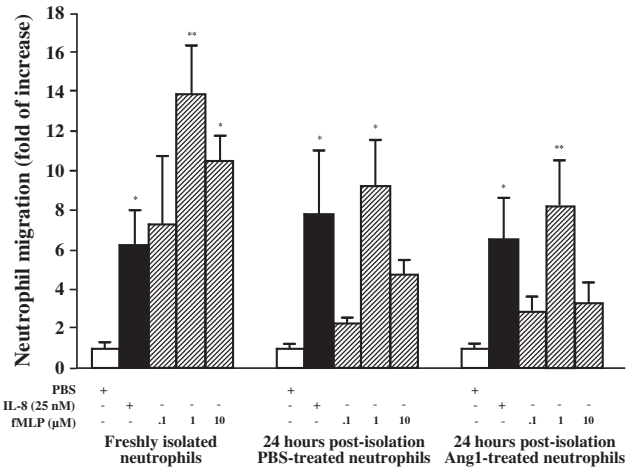

Fig. 9. Neutrophil chemotactic activity is maintained upon 24-hour post-isolation period. *In vitro* chemotactic assays were performed in Transwell-96 well permeable supports. The upper and lower parts of the wells were separated by a porous polycarbonate membrane filter (3 μm pore size). Briefly, the bottom wells were loaded with RPMI containing PBS, IL-8 (25 nM) or fMLP (0.1–10 μM). The upper wells were loaded with RPMI containing neutrophils. In function of the experiments, neutrophils were freshly added post-isolation (left panel) or pretreated for 24 h with PBS or Ang1 (10 nM) (middle and right panels respectively) prior to a 1-h chemotactic assay. At the end of the incubation period, the supernatant from the bottom wells was collected, and a count of migrating neutrophils was assessed with an Amnis ImageStream system. *$p<0.05$; **$p<0.01$ as compared to corresponding PBS-treated cells.

4.1. Differential effects of Ang1 and Ang2 on neutrophil viability

Ang1 and Ang2 have been reported to promote differential activities; Ang1 being recognized as a Tie2 receptor agonist, supporting the maturation and the stabilization of the neovessels, while Ang2 was recognized as an antagonist, promoting vascular permeability and the regression of pre-existing vasculature [3,4,14,15]. However, under specific conditions, Ang2 can also act as a Tie2 agonist by supporting Tie2 phosphorylation and associated downstream biological activities [16–19]. For instance, in ECs, Ang1 is recognized to inhibit apoptosis through the activation of PI3K/Akt and ERK1/2 pathways, which cooperate to prevent pro-apoptotic caspase-3, -7, and -9 activation [10,11]. For Ang2, the data are less clear; some groups reported that Ang2 has pro-apoptotic activities [55,56], whereas others reported that Ang2 could promote EC survival through the activation of ERK1/2 and PI3K pathways [17,57]. In addition, we reported that both angiopoietins share common agonistic pro-inflammatory activities, namely, on their capacity to promote endothelial PAF synthesis and P-selectin translocation [20–22]. In neutrophils, we reported the expression of Tie2 receptor, and the capacity of both angiopoietins to induce PAF synthesis, CD11/CD18 (β_2-integrin) activation, neutrophil adhesion and migration [22,24]. This latter effect was also confirmed by an independent group [65]. In addition, considering that both angiopoietins can modulate EC viability and induce the synthesis and release of a neutrophil pro-survival mediator, namely PAF [10,11,17,22,42,55–57], we were led to address the capacity of both angiopoietins to modulate neutrophil viability, and if so, define the role of PAF in that process.

In the current study, we performed a concentration- and time-dependent assay, and observed that Ang1 is providing a pro-survival activity on the neutrophils by delaying the entry of cells into apoptotic and necrotic phases, while Ang2 had no significant effect on basal viability. In addition, we observed that Ang1/Tie2 interaction is needed to support Ang1 pro-survival activity, as a pretreatment with selective blocking anti-human Tie2 antibodies, almost completely abrogated ($\approx$85%) Ang1 pro-survival activity in neutrophils. The residual effect could be due to an incomplete blockade of Tie2 receptor by the selective antibodies, thus, allowing partial Ang1/Tie2 interaction and biological activity. Another possibility could be that Ang1 can induce some biological activities upon its binding to specific integrins, independently from Tie2 participation [9,80,81]. If so, then it appears that Ang1/integrin interaction would have a minor contribution on Ang1 pro-survival activity.

4.2. Cooperation and contribution of PAF and IL-8 on Ang1-mediated pro-survival activity

In addition to the differential capacity of Ang1 to prolong the viability of the neutrophils, as opposed to Ang2, we recently observed another differential effect between Ang1 and Ang2 on neutrophils. Indeed, we reported that Ang1 (but not Ang2) promotes IL-8 synthesis and release from neutrophils [77]. Since IL-8 is a pro-survival mediator for the neutrophils [54], we hypothesized that the synthesis and/or the release of IL-8, might as well, like endogenous PAF supports the pro-survival activity of Ang1 on neutrophils. Our data demonstrate that a combination of PAF or IL-8 with Ang1 produced an additive effect on neutrophil viability. These results are in agreement with previous studies demonstrating that under *in vivo* pro-inflammatory environments, different mediators can cooperate to increase neutrophil viability, thereby delaying their entry into apoptotic phase, even up to several days [26] and thus, resulting in an enhanced inflammatory state which can become pathological [29].

To delineate the contribution of PAF on Ang1 pro-survival activity in neutrophils, we addressed the effect of a selective PAF receptor antagonist (BN 52021) [67,68] on Ang1 anti-apoptotic activity. Treatment with BN 52021 did not modulate basal neutrophil viability, whereas it was efficient to prevent pro-survival activity mediated by exogenous PAF. In addition, in pretreatment, BN 52021 did not inhibit Ang1 pro-survival activity on neutrophils, thus, suggesting that Ang1 anti-apoptotic activity is PAF-independent. We previously reported that Ang1 and Ang2 could induce PAF synthesis from ECs through the activation of $sPLA_2$-V, and independently from $cPLA_2$ participation [21]. We also observed that both angiopoietins could promote neutrophil PAF synthesis [22]. In neutrophils, both $cPLA_2$ and $sPLA_2$-V are expressed and can contribute to PAF synthesis in function of the stimuli [69,71–73]. Thereby, we addressed the effect of selective inhibitors of the secreted and cytosolic form of PLA_2 on Ang1-mediated pro-survival activity on neutrophils. Pretreatment of neutrophils either with the $cPLA_2$ inhibitor [74–76], $AACOCF_3$, or the $sPLA_2$ inhibitor [74,75,82], scalaradial, did not reduce Ang1 pro-survival activity on neutrophils. Consequently, if both angiopoietins (Ang1 and Ang2) can support the endogenous PAF synthesis from neutrophils [22], the concentration of PAF synthesized is not sufficient to participate to Ang1-mediated pro-survival activity on the neutrophils.

Since PAF cannot be considered as the pro-inflammatory mediator supporting Ang1-mediated pro-survival activity, we thus addressed whether IL-8 release was implicated in Ang1-induced neutrophil viability. Under basal condition, there is no or marginal release of IL-8 by the neutrophils, and a treatment with an anti-human IL-8 blocking antibodies did not modulate the basal survival rate. However, in pretreatment to Ang1-stimulation, the anti-IL-8 blocking antibodies provided a marked reduction ($\approx$80%) of Ang1 pro-survival effect. Consequently, these data are supporting our hypothesis that the synthesis and release of IL-8 plays a pivotal role as a discriminating factor for the differential capacity of Ang1, as opposed to Ang2, to support neutrophil viability. The incomplete reduction of Ang1-mediated pro-survival activity following a pretreatment with IL-8 blocking antibodies could suggest that Ang1 can activate downstream signaling pathways capable to provide pro-survival/anti-apoptotic activities, and/or that Ang1 is capable to promote the release of other pro-survival mediators.

We also reported that PAF is capable to promote IL-8 synthesis and release by the neutrophils [77]. Since a treatment with exogenous PAF is capable to promote neutrophil viability, we addressed the contribution of IL-8 on PAF pro-survival activity. By pretreating the neutrophils with anti-IL-8 blocking antibodies, we observed an obliteration of PAF anti-apoptotic activity. These latter observations demonstrate that the synthesis and release of IL-8 upon a stimulation of neutrophils either with exogenous Ang1 or PAF is the common factor responsible for their pro-survival activity.

It is well defined that transvascular migrated neutrophils are playing a major role in the development of pathological inflammation by prolonging their survival half-life, thus, extending their capacity to promote the synthesis and release of pro-inflammatory mediators, cytokines and metalloproteinases [28,29,83–85]. We and other groups reported the chemotactic activity of Ang1 and other cytokines on freshly isolated neutrophils [24,65,86]. Herein, we observed that a treatment of the neutrophils with Ang1 under acute or prolong time period did not alter the capacity of the neutrophils to migrate upon their stimulation by chemotactic mediators, such as fMLP and IL-8.

Previous studies reported that under *in vitro* conditions, Ang1 possesses anti-inflammatory properties. Ang1 reduces VEGF-induced leukocyte adhesion onto HUVEC and E-selectin expression [87] and thrombin-mediated neutrophil adhesion onto EC [88]. Other studies reported that under *in vivo* conditions, Ang1 has an anti-inflammatory role as observed in acute lung injury, endotoxic shock and cardiac remodeling [12,13,89]. Similarly, the genetic overexpression or overexpression following infection with adenovirus carrying the Ang1 gene did protect the vasculature from VEGF and irritant-induced leakage in mice [4,90]. On the other hand, we and other groups reported that under *in vitro* conditions Ang1 can have pro-

inflammatory activities [20–22,24,65,77,91]. In addition, it has also been reported that the administration of Ang1 did not inhibit VEGF-induced vascular permeability but delayed edema resolution, thus suggesting that Ang1 is not a universal inhibitor of EC permeability as previously thought [18]. Together, these observations could suggest that Ang1 may have differential biological activities in function of the biological conditions and environment.

In summary, our study is the first one to report that Ang1, upon its binding to Tie2 receptor, is providing a pro-survival activity on neutrophils, as opposed to Ang2, which has no effect. In addition, Ang1 pro-survival activity is mainly driven by its capacity to promote the synthesis and release of IL-8 by the neutrophils. However, additional studies will be needed to delineate which intracellular signaling pathways are participating in the induction of Ang1 pro-survival and anti-apoptotic activities on neutrophils. Together, our data provides novel insight in the capacity of Ang1 to prolong neutrophil viability and to promote the release of pro-inflammatory mediators, which could contribute to support the development of pathological vascular diseases associated to inflammatory response.

Supplementary data to this article can be found online at doi:10.1016/j.bbamcr.2011.12.002.

Abbreviations

Ang1	angiopoietin-1
Ang2	angiopoietin-2
ARDS	acute respiratory distress syndrome
$cPLA_2$	cytosolic phospholipase A_2
EC	endothelial cell
FITC	fluorescein isothiocyanate
fMLP	formyl-Methionyl-Leucyl-Phenylalanine
IL-8	interleukin-8
MAPK	mitogen-activated protein kinase
PAF	platelet activating factor
P.I.	propidium iodide
PKC	protein kinase C
SIRS	systemic inflammatory response syndrome
$sPLA_2$	secreted phospholipase A_2
TNF-α	tumor necrosis factor-α
VEGF	vascular endothelial growth factor

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ANNEXE 2

Article original qui sera publié dans Journal of Cellular Physiology (epub 2011 Oct 20).

Original Research Article

Angiopoietin-1 but not angiopoietin-2 induces IL-8 synthesis and release by human neutrophils

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Running head: Ang1-mediated IL-8 release by neutrophils

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ABSTRACT

We previously reported Tie2 receptor expression on human neutrophils, which promotes chemotactic activities upon activation by both angiopoietins (Ang1 and Ang2). Moreover, we observed that neutrophil pretreatment with Ang1 or Ang2 enhances IL-8 chemotactic effect. Therefore, we assessed the capacity of Ang1 and/or Ang2 to modulate neutrophil IL-8 synthesis and release. Neutrophils isolated from healthy donors were stimulated in a time- (1-6 hours) and concentration- (10^{-10}-10^{-8} M) dependent manner with both angiopoietins. IL-8 mRNA production was measured by RT-qPCR, whereas its protein synthesis and release from neutrophils was assessed by ELISA. Ang1 (10^{-8} M) induced a significant and maximal increase of IL-8 mRNA (4.7-fold) within 1 hour, and promoted maximal IL-8 protein synthesis (3.6-fold) and release (5.5-fold) within 2 hours as compared to control PBS-treated neutrophils. Treatment with Ang2 alone did not modulate IL-8 synthesis or release, and its combination to Ang1 did not affect Ang1 activity. Neutrophil pretreatment with a protein synthesis inhibitor (CHX) increased IL-8 mRNA synthesis by 18-fold, and reduced Ang1-mediated IL-8 protein synthesis and release by 96 and 92% respectively. Pretreatment with a transcription inhibitor (ActD) reduced IL-8 mRNA synthesis by 54% and IL-8 protein synthesis and release by 52 and 79% respectively. Using specific kinase inhibitors, we observed that Ang1-driven IL-8 mRNA and protein synthesis is p42/44 MAPK-dependent and -independent from p38 MAPK and PI3K activity. Our study is the first to report the capacity of Ang1 (as opposed to Ang2) to promote neutrophil IL-8 synthesis and release through the activation of p42/44 MAPK pathway.

INTRODUCTION

Angiogenesis is the process by which new blood vessels are developing from pre-existing vasculature. This process is timely regulated by the coordinated participation of selective growth factors, such as VEGF and both angiopoietins (Ang1 and Ang2) (Carmeliet, 2000; Jones et al., 2001; Yancopoulos et al., 2000). In addition, these growth factors are also involved in the regulation of inflammatory processes (Fiedler et al., 2006; Kanazawa, 2007; Kim et al., 2001; Roviezzo et al., 2005). The principal members of the angiopoietins, Ang1 and Ang2, were found to activate Tie2 receptor, initially reported for being expressed on endothelial cell (EC) surface. Ang1 has been characterized as Tie2 agonist, promoting the maturation of unstable vessels in the presence of VEGF (Thurston et al., 1999), whereas Ang2 was initially described as natural endogenous Tie2 antagonist for its capacity to destabilize preexisting vessels prior to VEGF-induced angiogenesis (Maisonpierre et al., 1997). However, over the last decade, mounting evidences indicated that Ang2 can also behave as Tie2 agonist, as demonstrated by its capacity to promote EC tube-like structure formation and migration (Mochizuki et al., 2002; Teichert-Kuliszewska et al., 2001) and EC survival (Harfouche and Hussain, 2006). Recent studies also revealed that angiopoietins can modulate EC behaviour toward proinflammatory cytokines. For instance, Ang2 was shown to serve as a priming factor for tumor necrosis factor (TNF)-α (Fiedler et al., 2006). Moreover, Ang1 and Ang2 were shown to potentiate VEGF-mediated angiogenesis in a mouse cornea model (Asahara et al., 1998).

Pathological angiogenesis is proactive in the development of diseases such as tumor growth metastasis, atherosclerosis, rheumatoid arthritis, psoriasis and vascular retinopathy (Carmeliet, 2005). One common trait between these pathological conditions is the presence of an inflammatory environment, which precedes and accompanies angiogenesis, as evidenced by a vascular permeability increase and the recruitment of inflammatory cells (Dvorak et al., 1995; Jackson et al., 1997).

Amongst inflammatory cells, neutrophils are the first ones to be recruited to the inflammatory site, which participate in the development of a pro-angiogenic environment by their capacity to synthesize and release pro-inflammatory and pro-angiogenic cytokines such as Ang1, VEGF, TNF-α, interleukins (ILs; IL-1, IL-6 and IL-8) and matrix metalloproteinase degrading enzymes (MMPs) which are all contributing to orchestrated sequences associated to blood vessels formation (McCourt et al., 1999; Shaw et al., 2003).

Interleukin-8 (IL-8) is one of the major pro-inflammatory mediators released by the neutrophils inducing numerous inflammatory activities such as respiratory burst (Schroder et al., 1987), generation of superoxide and hydrogen peroxide (Thelen et al., 1988), cellular reorganization (Thelen et al., 1988), secretion of catalytic enzymes (metalloproteinases, lysosymes) (Peveri et al., 1988), synthesis of bioactive lipids (Schroder, 1989), translocation and activation of adhesion molecules (Paccaud et al., 1990), chemotaxis (Huber et al., 1991) and pro-survival activity (Goodman et al., 1998). In addition, in function of the stimuli the release of IL-8 by the neutrophils is dependent on the activation of p38 and/or p42/44 MAPK pathways (Lee et al., 2009; Marie et al., 1999). On our side, we observed that Ang1 and Ang2 mediate neutrophil migration in a concentration-dependent manner, and that a pre-treatment of the neutrophils either with Ang1 or Ang2 increase IL-8 chemotactic activity. These latter effects were mediated through the activation of the PI3K/Akt pathway and independently from p38 and p42/44 MAPK (Brkovic et al., 2007).

Since neutrophils express IL-8 constitutively, and that its release can be triggered upon stimulation by selective pro-inflammatory mediators, we wanted to assess the capacity of both angiopoietins to promote IL-8 synthesis and release from the neutrophils, and if so, to delineate the signalling pathways involved in these processes.

MATERIAL AND METHODS

Neutrophil isolation and purification

Venous blood was obtained from healthy donors free from medication for at least 10 days before the start of the experiments. The study has been approved by the human ethical committee of the Montreal Heart Institute, and all subjects provided written informed consent. Neutrophils were isolated as described previously (Brkovic et al., 2007). Ninety-eight percent of the isolated cells were polymorphonuclear cells, as determined with a Coulter counter, and viability was found to be greater than 98%, as assessed by Trypan blue dye exclusion assay.

Neutrophil stimulation for IL-8 quantification

Purified neutrophils (5 x 10^6/ml; 500 µl) were incubated in RPMI-1640 solution (Cambrex BioScience, Walkersville, MD) supplemented with 5% fetal bovine serum (FBS) (PAA Laboratories, Etobicoke, ON), 1% penicillin/streptomycin/GlutaMAX (P/S) (GIBCO, Grand Island, NY) and 25 mM HEPES (Sigma, Oakville, ON) and termed RPMI* (for complete RPMI-1640 solution).

In a first series of experiments, neutrophils were stimulated from 1 to 6 hours with N-Formyl-Met-Leu-Phe (fMLP), bacterial lipopolysaccharide (LPS; *E. Coli* 0111:B4), (Sigma), Ang1 and Ang2 (R&D Systems, Minneapolis, MN) at 37°C, 5% CO_2. Upon neutrophil stimulation, cells were centrifuged at 900 g for 7 minutes and supernatants stored at -80°C for future IL-8 quantification by ELISA development kits (R&D Systems) accordingly to manufacturer's instructions and performed on high-binding specific Immulon® 4 HBX 96-well plates. In another set of experiments, neutrophils (5 x 10^6/ml; 500 µl) were pretreated with either an mRNA transcription inhibitor (Actinomycin D; Sigma) or a protein synthesis inhibitor (Cycloheximide; Calbiochem, La Jolla, CA) for 30 minutes prior to agonist stimulation for 1 to 6 hours as described above. Upon neutrophil stimulation, cells were centrifuged at 900 g for 7 minutes and supernatants were stored at -80°C for IL-8 quantification as aforementioned.

Neutrophil pellets were vortexed for 30 seconds after being resuspended in RPMI*- containing 1% Triton (J.T. Baker; Phillipsburg, NJ) and a protease inhibitor (Pefabloc, 1 mM; Sigma) -solution in order to collect intracellular neutrophil content. Cells were then centrifuged at 15000 g for 7 minutes at 4°C, and supernatants stored at -80°C for IL-8 quantification by ELISA.

In the last series of experiments, neutrophils (5 x 10^6/ml; 500 µl) were pretreated with specific inhibitors of p38 MAPK (SB203580; Calbiochem), MEK-1/2 (U0126; Millipore, Billerica, MA), Akt (Triciribine; Calbiochem) and with normal goat IgG or goat anti-human Tie2 IgG (R&D Systems) for 30 minutes prior to agonist stimulation. Upon neutrophil stimulation, cells were centrifuged at 900 g for 7 minutes. Supernatants were stored at -80°C for further IL-8 quantification by ELISA. In order to collect neutrophil intracellular content, cell pellets were vortexed for 30 seconds after being resuspended in RPMI-1640*- containing 1% Triton and Pefabloc. Cells were then centrifuged at 15000 g for 7 minutes at 4°C, and supernatants stored at -80°C for further IL-8 quantification by ELISA.

ERK-1/2 phosphorylation analyses

Neutrophils (5 x 106/ml) were stimulated with fMLP, LPS and Ang1 at 37°C, 5% CO2 for 5 to 60 minutes as described above. In parallel, a series of experiments were carried by pretreating the neutrophils with the MEK-1/2 inhibitor (U0126) for 30 minutes prior to agonist stimulation for an additional 30 minutes. At the end of each incubation time period, neutrophils were centrifuged at 900g for 7 minutes at 4°C and the supernatant discarded. Neutrophil pellets were vortexed for 30 seconds after being resuspended in lysis buffer (1 mM EDTA, 0.5% Triton X-100, 5 mM NaF, 6 M urea, 10 µg/ml Leupeptin, 10 µg/ml Pepstatin, 100 µM PMSF, 3 µg/ml Aprotinin, 2.5 mM sodium pyrophosphate, 1 mM activated sodium orthovanadate in PBS, pH 7.4) and incubated for 15 minutes on ice. Cells were then centrifuged at 15000 g for 7 minutes at 4°C and supernatants stored at -80°C for further quantification of ERK phosphorylation by the human/mouse/rat phospho-ERK1

(T202/Y204)/ERK2 (T185/Y187) ELISA kit (R&D Systems) accordingly to manufacturer's instructions.

Real time quantitative transcriptase-polymerase chain reaction (RT-qPCR) analyses

Neutrophils (5 x 10^6/ml; 1 x 10^7 total) were stimulated (1 to 6 hours) as described above. Total RNAs were isolated by using RNeasy extraction kit (Qiagen, Mississauga, ON, Canada). Seventy-five nanogram of RNAs was reverse transcribed using random hexamers and the Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen, Burlington, ON, Canada) as described by the manufacturer. Primers were used to amplify a 143-base pair (bp) fragment of IL-8 cDNA (GenBank no. NM_000584.2; forward: 5'-GCAGCTCTGTGTGAAGGTGCAGT-3', and reverse 5'-GTGTTGGCGCAGTGTGGTCC -3') and a 93-bp fragment of β-microglobulin cDNA (GenBank no. MIM109700; forward: 5'-TAGCTGTGCTCGCGCTACTC -3', and reverse: 5'-TTCCATTCTCTGCTGGATGACG-3'). The expression level of IL-8 was determined using a Brilliant SYBR Green qPCR master mix (Invitrogen) and a Mx3000P real-time PCR system (Stratagene). To confirm the specificity of the PCR products, the melting profile of each sample was determined by heating from 55°C to 95°C while measuring the fluorescence emitted. Analysis of the melting curve showed that each pair of primers amplified a single product. Each run consisted of an initial denaturation time of 10 min at 95°C and 40 cycles at 95°C for 30 s, 55°C for 60 s, and 72°C for 60 s.

Statistical analysis

Results were expressed as the mean ± SEM. Comparisons were made by analysis of variance (ANOVA) followed by a Bonferroni *t*-test. Differences were considered significant at p values ≤ 0.05.

RESULTS

Angiopoietins effect on IL-8 release by the neutrophils

In a first series of experiments we assessed the capacity of angiopoietins to induce IL-8 release from the neutrophils in a concentration- (10^{-10} – 10^{-8} M) and time- (1 – 6 hours) dependent manner. We also used positive controls known for their capacity to promote neutrophil IL-8 release, such as fMLP (10^{-7} M) and LPS (10 µg/ml) (Cassatella et al., 1992; Fujishima et al., 1993). Treatment with Ang1 at 10^{-10} and 10^{-9} M for up to 6 hours did not mediate IL-8 release from neutrophils (**Fig. 1**). However, a treatment with Ang1 at 10^{-8} M increased significantly IL-8 release within 1 hour and the maximal effect (5.6-fold increase) was achieved within 2 hours as compared to PBS-treated cells. Treatment with Ang2 (10^{-10} – 10^{-8} M) for up to 6 hours did not mediate IL-8 release from neutrophils. To address whether the addition of Ang2 might affect Ang1-mediated IL-8 release, we combined both angiopoietins (10^{-10} – 10^{-8} M), and the results were similar to those mediated by Ang1 alone. All the positive controls tested did induce a significant increase of IL-8 release as compared to PBS-treated cells from 1 to 6 hours post-treatment. The most powerful agonist being LPS, which increased IL-8 release by up to 33-fold within 6 hours as compared to PBS-treated cells whereas the fMLP-treated cells induced a 13-fold increase of IL-8 release after 4 hours of stimulation (**Fig. 1**).

Ang1 effect on IL-8 protein and mRNA synthesis in the neutrophils

Although small concentrations of IL-8 protein are found in resting post-isolated neutrophils, the majority of IL-8 released comes from *de novo* synthesis (Kuhns and Gallin, 1995; Lund and Øesterud, 2004). Therefore, we assessed the capability of Ang1 to induce neutrophil IL-8 synthesis. Upon neutrophil isolation, the constitutive endogenous concentration of IL-8 was 0.25 ± 0.04 ng/5 x 10^6 neutrophils. Neutrophils were then stimulated as aforementioned to address the kinetic (0-6 hours) of IL-8 synthesis (**Fig. 2A**). Under physiological condition (PBS treatment), neutrophils had a constitutive IL-8 protein

synthesis rate ranging from 1.06 to 1.99 ng/5 x 10^6 neutrophils/hour from 1 to 6 hours post-isolation (**Fig. 2A**). Neutrophil incubation with fMLP and LPS promoted a rapid and transient rise in IL-8 protein synthesis. For both positive agonists, the maximal kinetic of IL-8 synthesis rate was observed within the first hour (10.15 and 9.80 ng/5 x 10^6 neutrophils/hour for fMLP and LPS), corresponding to a significant 9.6- and 9.2-fold increase respectively as compared to PBS-treated neutrophils. The induction of IL-8 protein expression mediated by fMLP and LPS returned to the basal *de novo* synthesis rate values within 2, 4 and 6 hours respectively (**Fig. 2A**). In fact, following the peak of IL-8 protein synthesis rate observed under fMLP stimulation, the synthesis rate of IL-8 was significantly decreased and even below the basal rate observed in PBS-treated neutrophils. Treatment with Ang1 (10^{-9} M) presented a similar pattern of IL-8 synthesis rate as observed under PBS-treated neutrophils. However, at the higher concentration, Ang1 (10^{-8} M) increased the kinetic rate of IL-8 protein synthesis, by a maximum of 5.87 ng/5 x 10^6 neutrophils/hour within the first hour, corresponding to a significant 5.5-fold increase as compared to PBS-treated neutrophils (**Fig. 2A**).

Since IL-8 protein synthesis can derive from the translation of constitutive and/or *de novo* IL-8 mRNA, we measured the effect of positive controls and Ang1 on IL-8 mRNA synthesis in neutrophils. The basal level of IL-8 mRNA was quantified at each time point, the neutrophils treated with PBS were used as basal internal control and its relative value was set to 1 for each time point. Treatment with fMLP, LPS and Ang1 (10^{-8} M) promoted a rapid and significant increase (within the first hour) of IL-8 mRNA content by 4.7-, 4.6- and 4.1-fold respectively as compared to PBS-treated neutrophils (**Fig. 2B**). Upon the first hour of stimulation, fMLP and Ang1 (10^{-8} M) were no longer capable to modulate positively the DNA transcription into IL-8 mRNA. On the other hand, under LPS challenge, we observed up to 4 hours, a sustained high level of IL-8 mRNA expression (**Fig. 2B**).

Within the same series of experiments, we also addressed the capacity of Ang2 (10^{-9} and 10^{-8} M) to promote IL-8 protein and mRNA synthesis, and the latter had no significant effect as compared to

control PBS-treated cells (data not shown). By integrating the data from figures 1 and 2A, we observed that under basal conditions, about 0.5% of total IL-8 synthesized was released by the neutrophils within a 6-hour time period, whereas neutrophils stimulation either with fMLP, LPS or Ang1 (10^{-8} M) provided a significant increase of IL-8 release by 9.0-, 12.4-, and 5.3-fold respectively as compared to PBS-treated cells (Table I).

Effect of CHX on IL-8 mRNA and protein expression in neutrophils

Ang1 (10^{-8} M) and the positive controls induce a rapid and maximal IL-8 mRNA and protein synthesis within the first hour post-stimulation, which can be sustained for up to 4 hours under LPS stimulation. Thus, we wanted to verify if this rapid *de novo* IL-8 protein synthesis affects the outcome of IL-8 mRNA and protein synthesis. To do so, we used cycloheximide (CHX; 10 μg/ml), a broad eukaryotic translation elongation inhibitor, which prevents protein synthesis deriving from mRNA (Schneider-Poetsch et al., 2010).

Pretreatment with CHX increased significantly IL-8 mRNA synthesis, up to 3.8-fold in control PBS-treated neutrophils from 1 to 6 hours (**Fig. 3A**). The addition of CHX significantly upregulated IL-8 mRNA synthesis up to 2 hours after fMLP, LPS and Ang1 (10^{-8} M) treatment by a maximum of 7.3-, 5.1- and 8.6-fold compared to vehicle-DMSO treated neutrophils, and this latter effect faded with time towards basal levels (**Fig. 3A**).

Pretreatment with CHX inhibited the basal rate of IL-8 protein synthesis (PBS-treated cells) by up to 74% for the 6-hour period and was efficient to abrogate by 94, 94 and 91% the acute synthesis rate of IL-8 protein observed within the first hour upon stimulation with fMLP, LPS and Ang1 (10^{-8} M) respectively (**Fig. 3B**). Since no significant IL-8 protein synthesis rate occurred following the one-hour peak, CHX did not further modulate the IL-8 protein synthesis rate from 2 to 6 hours post-stimulation (**Fig. 3B**).

In addition, we observed that a pretreatment with CHX, reduced the basal IL-8 release by up to 92% after 1 hour incubation. Similarly, CHX inhibited IL-8 release mediated by fMLP, LPS or Ang1 (10^{-8} M) with maximal inhibitions of 93, 93 and 92% respectively, at one hour post-treatment and the inhibitory capacity of CHX was maintained across the 6-hour time period of the experiment (**Fig. 3C**).

Effect of ActD on IL-8 mRNA and protein expression in neutrophils

Since we observed that Ang1 (10^{-8} M) and both positive controls (fMLP and LPS) can induce the synthesis of IL-8 mRNA and its protein in the neutrophils, we addressed the capacity of Ang1 to modulate IL-8 mRNA stability and delineate the origin of its protein (constitutive or *de novo* synthesis). Thus, we investigated the effects of an mRNA synthesis inhibitor, namely actinomycin D (ActD; 5 μg/ml) on Ang1-induced IL-8 mRNA and protein synthesis.

Pretreatment with ActD reduced gradually and significantly the basal IL-8 mRNA synthesis over time, reaching a maximal inhibition of 82% after 6 hours (**Fig. 4A**). Pretreatment with ActD decreased the IL-8 mRNA synthesis induced by fMLP and LPS (1-6 hours), with a maximal inhibition of 85 and 93 % upon a 6 hour-incubation time period. However, ActD was efficient to decrease Ang1 (10^{-8} M)-mediated IL-8 mRNA synthesis by 67% and only within the first hour post-stimulation (**Fig. 4A**).

Pretreatment with ActD inhibited in a time-dependent manner (1 to 6 hours) the basal rate of IL-8 protein synthesis by up to 100% (**Fig. 4B**). The incubation with ActD inhibited IL-8 synthesis rate induced by fMLP, LPS and Ang1 (10^{-8} M) between 0-1 hour by 38, 41 and 39% respectively. As previously described in the CHX study, following the initial peak of IL-8 protein synthesis (from 0-1 hour), the IL-8 synthesis rate decreased over time and the pretreatment with ActD was no longer or less effective at decreasing the rate of IL-8 protein synthesis (**Fig. 4B**).

Using the supernatant collected from the neutrophils treated for the quantification of IL-8 protein synthesis, we tested the capacity of ActD to modulate IL-8 release. Pretreatment with ActD, reduced the

basal IL-8 release by up to 62% after 6 hours post-incubation (**Fig. 4C**). Similarly, ActD inhibited IL-8 release mediated by fMLP and LPS with maximal inhibitions of 79 and 73% at 6 hours post-stimulation, and the inhibitory capacity of ActD was maintained across the 6-hour time period of the experiment. In addition, ActD pretreatment reduced Ang1 (10^{-8} M)-mediated IL-8 protein release at 1 hour post-treatment by 72% respectively (**Fig. 4C**).

Effect of selective kinase inhibitors on IL-8 mRNA and protein modulation in neutrophils

In order to delineate the pathways involved in Ang1-induced IL-8 mRNA and protein synthesis, we targeted the contribution of selective protein kinases (PI3K, p38 and p42/44 MAPK) involved in these processes. Since IL-8 mRNA synthesis was maximal within 1 hour of stimulation with Ang1 (10^{-8} M), we used the same time period to determine if the aforementioned kinases play a role in Ang1-induced IL-8 mRNA synthesis. Neutrophils were incubated with the vehicle (DMSO), a p38 MAPK inhibitor (SB203580; 10 μM) (Cuenda A Fau - Rouse et al.), a MEK-1/2 inhibitor (U0126; 20 μM) (Favata et al., 1998) or a PI3K inhibitor (triciribine; 5 μM) (Sun et al., 2009) 30 minutes prior to agonist stimulation for one hour. Neutrophil treatment with all kinase inhibitors alone did not modulate the basal IL-8 mRNA synthesis (**Fig. 5A**). Incubation with SB203580 increased significantly the synthesis of IL-8 mRNA induced by fMLP and Ang1 (10^{-8} M) by 102 and 94% respectively, whereas LPS-induced IL-8 mRNA synthesis was reduced by 61%. On the other hand, treatment with U0126 completely abrogated IL-8 mRNA synthesis stimulated by fMLP and Ang1 (10^{-8} M), without affecting LPS-induced IL-8 mRNA synthesis. Finally, the blockade of PI3K pathway with triciribine did not modulate significantly the expression of IL-8 mRNA induced by both positive agonists and Ang1 (10^{-8} M) (**Fig. 5A**).

Pretreatment with each kinase inhibitor did not modulate the basal IL-8 protein synthesis after one hour of incubation. Moreover, pretreatment with the p38 MAPK inhibitor (SB203580) at the highest concentration (10 μM), only inhibited significantly LPS-induced IL-8 protein synthesis by 53%,

without affecting IL-8 protein synthesis mediated by fMLP and Ang1 (10^{-8} M) (**Fig. 5B**). On the other hand, a 30 minutes incubation period with U0126 (20 μM) prior to agonist stimulation inhibited significantly fMLP and Ang1 (10^{-8} M)-driven IL-8 protein synthesis by 83 and 94% respectively, while U0126 had no effect on IL-8 protein synthesis mediated by LPS stimulation. Pretreatment with the Akt inhibitor (triciribine) did not provide significant effect on IL-8 protein synthesis mediated by all the agonists including Ang1 (**Fig. 5B**).

Finally, we assessed the contribution of these protein kinases on IL-8 release by the neutrophils upon stimulation by both controls and Ang1. As the plateau of IL-8 release mediated by Ang1 is observed within 2 hours post-stimulation, we elected this time period in the current series of experiments. Individual pretreatment of neutrophils with the kinase inhibitors did not modulate the basal release of IL-8 upon 2 hours of incubation. A pretreatment for 30 minutes with SB203580 (10 μM) prior to agonists stimulation reduced significantly LPS-induced IL-8 release by 83% while having no effect on fMLP and Ang1 (10^{-8} M)–mediated IL-8 release (**Fig. 5C**). Pretreatment with the MEK 1/2 inhibitor U0126 (20 μM) inhibited fMLP- and Ang1-induced neutrophil IL-8 release by 83 and 95% respectively, without affecting LPS-mediated IL-8 release by the neutrophils. Finally, the blockade of the PI3K pathway with the Akt inhibitor triciribine (5 μM) did not interfere with IL-8 release mediated by fMLP, LPS and Ang1 (10^{-8} M) (**Fig. 5C**).

Ang1 mediated ERK-1/2 phosphorylation in neutrophils

In order to confirm that the inhibitory effect of U0126 on IL-8 mRNA and protein synthesis mediated by fMLP and Ang1 in the neutrophils is due to its capacity to block ERK-1/2 phosphorylation (p-ERK-1/2), we investigated the capacity of fMLP (10^{-7} M) and Ang1 (10^{-8} M) to phosphorylate ERK-1/2 from 5 to 60 minutes (**Fig. 6A**). The basal level (PBS-treatment) of p-ERK-1/2 was used as basal internal control and its relative value was set to 1 for each time point. Treatment with fMLP induced a significant rapid

and sustained phosphorylation of ERK-1/2 (from 5 to 60 minutes), which was maximal (7.9-fold increase) within 30 minutes post-stimulation, whereas Ang1 was capable to significantly increase ERK-1/2 phosphorylation at 15 and 30 minutes post-stimulation, the latter time point providing a 2.6-fold increase as compared to PBS-treated neutrophils (**Fig. 6A**).

Since the most efficient ERK-1/2 phosphorylation increase was achieved after 30 minutes for fMLP and Ang1, we selected this time period to determine the efficiency of the MEK-1/2 inhibitor (U0126) to prevent ERK-1/2 phosphorylation. Herein, we pretreated the neutrophils with U0126 (2 and 20 μM) or the vehicle (DMSO) for 30 minutes before their incubation with both agonists for an additional 30 minutes. Firstly, we observed that a pretreatment with the vehicle (DMSO) did not affect the phosphorylation of ERK-1/2 mediated by PBS, fMLP and Ang1 (**Fig. 6B**). However, a pretreatment with U0126 at the lowest concentration (2 μM) inhibited ERK-1/2 phosphorylation induced by fMLP and Ang1 by 86 and 87% respectively, and at the highest concentration, U0126 (20 μM) completely abrogated fMLP- and Ang1-induced ERK-1/2 phosphorylation (**Fig. 6B**).

DISCUSSION

This study addresses for the first time the capacity of angiopoietins to induce the expression of IL-8 mRNA, its protein synthesis and release by the neutrophils. Interestingly, these effects are only mediated by Ang1, thus, suggesting a distinct agonistic capacity between Ang1 and Ang2 on neutrophils. Using selective inhibitors we observed that Ang1 induces these events through the activation of p42/44 MAPK pathway.

Differential effects of Ang1 and Ang2 on IL-8 protein synthesis and release

First, we assayed the capacity of the angiopoietins to induce the release of IL-8 by the neutrophils and we observed that only Ang1 at the highest concentration was capable to induce a rapid and transient IL-8 release. Interestingly, Ang2 had no effect on neutrophil IL-8 protein synthesis and release. This observation is in line with a parallel study in which we observed that neutrophil survival is enhanced by Ang1 but not Ang2, and through its capacity to promote IL-8 release (Dumas et al., personal communication). These data are providing novel insight on the mounting evidences that Ang1 is capable to provide pro-inflammatory activities (Abdel-Malak et al., 2008; Ahmad et al., 2010; Brkovic et al., 2007; Harfouche et al., 2003; Lemieux et al., 2005; Maliba et al., 2006).

Protein synthesis in the neutrophils is occurring during different stages of their maturation (Borregaard et al., 2001), thus conferring them the capacity to support a rapid constitutive release of cytokines upon their recruitment to inflammatory sites. However, in some specific cases, inflammatory stimuli can induce an acute protein synthesis of selective cytokines in mature neutrophils (Kuhns et al., 2001; Kuhns et al., 1998; McColl et al., 1992). Upon neutrophil isolation, the basal constitutive content of IL-8 protein was marginal. However, under basal condition during the 6-hour incubation period, we observed a constant and sustained rate of IL-8 protein synthesis, and despite this marked increase of IL-8 protein content, there was only a very small fraction (<0.5%) of *de novo* synthesized IL-8 being released

by the neutrophils (Table I). This phenomenon can be explained by the fact that upon their isolation, neutrophils are lacking their physiological interaction with endothelial cells, which is known to increase basal IL-8 protein synthesis without promoting its release (Kuhns and Gallin, 1995). In our study, we observed that a treatment with Ang1 (10^{-8} M) and fMLP induced a very rapid (within 1 hour) and robust IL-8 protein synthesis, whereas a treatment with LPS promoted an acute and sustained rate of IL-8 protein synthesis. These latter observations are in agreement with previous studies (Arnold R Fau - Werner et al.; Cassatella et al., 1992; Fujishima et al., 1993; Marie et al., 1999; Taieb et al., 2002).

Ang1 induces IL-8 mRNA expression and stability in the neutrophils

Cytokine synthesis and release by neutrophils depend on preformed intracellular pools that are ready to be released upon inflammatory stimuli. However, in some specific cases, neutrophils are capable to induce mRNA transcription (Fessler et al., 2002; Kobayashi et al., 2002; Lindemann et al., 2004). Herein, stimulation of neutrophils by Ang1 and the positive controls enhanced IL-8 mRNA expression in a rapid and acute manner similarly to IL-8 protein synthesis and release. Following this rapid onset, IL-8 mRNA expression induced by Ang1 and fMLP was rapidly degraded, declining to basal levels. However, a treatment with LPS provided a sustained high level of mRNA expression throughout the 6-hour period, which correlates with its sustained capacity to promote IL-8 protein synthesis. Together, these observations confer to Ang1 similar agonistic properties as fMLP (moderate agonist) as compared to LPS (strong agonist) to support IL-8 mRNA expression, protein synthesis and release.

The IL-8 gene transcription regulation in various cell types is modulated by DNA-binding transcription factors that are acting as inducers (NF-kB, AP-1, CBP/p300) (Schmitz et al., 2001; Vanden Berghe et al., 1999; Whitmarsh and Davis, 1996) or repressors (NRF, OCT-1) (Nourbakhsh et al., 2001; Wu et al., 1997) of IL-8 mRNA synthesis. Moreover, IL-8 mRNA expression is maximal when a multiple complex including both types of regulators is formed (Carey, 1998). Under basal conditions,

the role of NRF is to silence IL-8 transcription, thus keeping its protein synthesis and release to low levels (Nourbakhsh et al., 1993). In our study, we observed an induction of IL-8 mRNA expression in PBS-treated neutrophils incubated with a protein synthesis inhibitor (CHX). Moreover, treatment with Ang1 and the positive controls in presence of CHX induced an even more robust overexpression of IL-8 mRNA by the neutrophils. The mRNA overexpression by protein synthesis inhibitors in presence or absence of agonists is known as superinduction (Herschman, 1991). The latter, can be triggered by various mechanisms including the stabilization of mRNA, stimulation of intracellular signaling, increased gene transcription and by modifying transcription factors activity (Edwards and Mahadevan, 1992). In our study, the IL-8 mRNA superinduction could be explained by the capacity of CHX to prevent *de novo* NRF protein synthesis, thus, preventing its capacity to silence IL-8 DNA transcription.

By incubating the neutrophils with a transcription inhibitor (ActD), we were capable to determine if the IL-8 mRNA synthesized is either degraded or stabilized during the 6-hour period. Treatment with ActD led during the 6-hour time period to the decay of IL-8 mRNA overexpressed under fMLP and LPS regimen. At the opposite, the overexpression of Ang1-induced IL-8 mRNA synthesis in presence of ActD was reduced only during the first hour post-stimulation, while during the remaining time period, the IL-8 mRNA content remained stable. This latter observation, demonstrate that Ang1 possesses the capacity to induce IL-8 mRNA stabilization in neutrophils.

Ang1 modulates IL-8 protein synthesis and release through enhanced translational activity

In order to determine if Ang1-induced IL-8 protein synthesis and release is modulated through transcriptional and/or translational mechanisms, we addressed the effects of CHX and ActD on IL-8 protein synthesis and release by the neutrophils. As expected, a pretreatment with protein synthesis inhibitor (CHX) completely abrogated the acute peak of IL-8 protein synthesis induced by Ang1 and positive agonists, subsequently leading to a signification reduction of the cumulative IL-8 protein

content upon the 6-hour stimulation period. In addition, we observed that CHX not only did prevent IL-8 protein synthesis but also reduced significantly the release of IL-8 by the neutrophils. These data demonstrate that IL-8 release by the neutrophils does not only originate from the depletion of endogenous pools of IL-8 but also requires *de novo* IL-8 protein synthesis.

Neutrophil treatment with a transcription inhibitor (ActD) partially reduced the initial peak of IL-8 protein synthesis induced by positive controls (fMLP and LPS). However, ActD pretreatment followed by Ang1 stimulation, modulated the initial IL-8 protein synthesis peak similarly to the positive agonists. Our data show that the initial peak IL-8 protein synthesis observed upon stimulation by Ang1 and positive agonists is driven by a combination of *de novo* IL-8 DNA to mRNA transcription and mRNA to protein translation. However, since a treatment with Ang1 as opposed to fMLP and LPS is capable to maintain IL-8 mRNA stability, this could explain why we observed a maintained synthesis of IL-8 protein in presence of ActD.

Ang1-mediated IL-8 mRNA and protein synthesis is driven by p42/44 MAPK activation

In function of the cells and agonists used, IL-8 mRNA expression, protein synthesis and release can be induced through the activation of p38 MAPK (Hashimoto et al., 1999; Islam et al., 2006), p42/44 MAPK (Neff et al., 2003; Shinkai et al., 2006) or PI3K/Akt (Tanaka et al., 2008; Tong et al., 2008). In a recent study, we observed that Ang1 induces neutrophil chemotaxis through the activation of PI3K/Akt pathway, and independently from p38 and p42/44 MAPKs (Brkovic et al., 2007). Herein, we observed that upon Ang1 or fMLP treatment, the IL-8 mRNA expression, protein synthesis and release were completely blocked by U0126, a MEK-1/2 inhibitor which prevents p42/44 MAPK phosphorylation (Favata et al., 1998), while it had no effect on LPS-treated neutrophils. Inversely, pretreatment with SB203580, a p38 MAPK inhibitor, did inhibit IL-8 mRNA expression, protein synthesis and release induced by LPS, while it had no inhibitory effect on Ang1 and fMLP-treated neutrophils. Finally,

pretreatment with triciribine, an inhibitor known to completely abrogate Akt phosphorylation even at concentrations lower than 5 μM (Miyamoto et al., 2008; Sun et al., 2009) had no effect on neutrophil IL-8 mRNA expression, protein synthesis and release induced by Ang1 and the positive agonists.

These data suggest that Ang1 and fMLP share a similar capacity to promote IL-8 mRNA expression, protein synthesis and release through the activation of p42/44 MAPK pathway, whereas LPS, a stronger inducer, appears to promote its activity through the activation of p38 MAPK. Interestingly, the blockade of p38 MAPK activation promoted the overexpression of IL-8 mRNA mediated by Ang1 and fMLP-treated neutrophils, suggesting a seesaw crosstalk between p38 and p42/44 MAPK, a well known phenomenon which has been observed to play a role in numerous biological activities and in a variety of cell types (Bellei et al., 2010; Kim et al., 2004; Shimo et al., 2007)

In summary, this study is the first one to demonstrate that Ang1 induces IL-8 mRNA expression, protein synthesis and release, as opposed to Ang2 which has no effect on these activities. Moreover, Ang1-induced IL-8 protein synthesis and release in the neutrophils is dependent on IL-8 mRNA expression followed by its stabilization and these latter phenomenon are regulated through p42/44 MAPK activation. Our study is supporting a recent finding in which we observed that Ang1 (but not Ang2) promotes neutrophil survival through the contribution of IL-8 release, which can contribute to prolong neutrophil pro-inflammatory activities.

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FIGURE LEGENDS

Figure 1: Effect of angiopoietins on IL-8 release by the neutrophils. Neutrophils (5 x 10^6/ml) were incubated for 1 to 6 hours with the vehicle (PBS) or various known IL-8 release inducers, namely fMLP (10^{-7} M) and LPS (10 µg/ml). Stimulation with Ang1 (10^{-10} – 10^{-8} M), Ang2 (10^{-10} – 10^{-8} M) or Ang1 + Ang2 (10^{-10} – 10^{-8} M) was also performed and the IL-8 released in the supernatant was quantified by ELISA. Results are expressed as the mean ± SEM of at least three independent experiments. Statistical comparisons were performed by analysis of variance (ANOVA) followed by a Bonferroni *t*-test. *p< 0.05, **p< 0.01 and ***p< 0.001 as compared to PBS-treated neutrophils at corresponding time points.

Figure 2: Effect of angiopoietins on IL-8 protein and mRNA synthesis by the neutrophils. Neutrophils (5 x 10^6/ml; 500 µl) (A) and (10^7/ml; 1 ml) (B) were stimulated with the vehicle (PBS), fMLP (10^{-7} M), LPS (10 µg/ml) and Ang1 (10^{-9} and 10^{-8} M) from 1 to 6 hours. Cell supernatant was removed and the neutrophil pellet was lysed in RPMI* + 1% Triton X100. Synthesized IL-8 in the neutrophils was quantified by ELISA. Synthesis rate represents the quantity of IL-8 protein synthesized per hour by the neutrophil between each time point (A). Total mRNA was isolated using the RNeasy extraction kit and converted to cDNA by RT, while IL-8 mRNA was quantified by RT-qPCR. The PBS value at each time point was set to one and IL-8 mRNA fold of increase was compared to the normalized PBS at corresponding time points (B). Results were expressed as the mean ± SEM of at least three independent experiments. Statistical comparisons were performed by analysis of variance (ANOVA) followed by a Bonferroni *t*-test. *p< 0.05, **p< 0.01 and ***p< 0.001 as compared to the respective PBS.

Figure 3: Effect of CHX on IL-8 mRNA expression, IL-8 protein synthesis and release by the neutrophils. Neutrophils were incubated either with the vehicle (DMSO) or CHX (10 μg/ml) for 30 minutes before the addition of PBS, fMLP (10^{-7} M), LPS (10 μg/ml) and Ang1 (10^{-9} and 10^{-8} M) for a period of 1 to 6 hours. IL-8 mRNA was quantified by RT-qPCR, whereas IL-8 protein quantification was performed as described in figures 1 and 2. For the IL-8 mRNA study, the PBS value at each time point was set to one and IL-8 mRNA fold of increase was compared to the normalized PBS at corresponding time points (A). Synthesis rate represents the quantity of IL-8 protein synthesized per hour by the neutrophil between each time point (B). IL-8 protein release is the quantity of IL-8 released at corresponding time points (C). Results were expressed as the mean ± SEM of at least three independent experiments. Statistical comparisons were performed by an analysis of variance (ANOVA) followed by a Bonferroni *t*-test. *p< 0.05, **p< 0.01 and ***p< 0.001 as compared to the respective PBS ± CHX pretreated neutrophils and †p< 0.05, ††p< 0.01 and †††p< 0.001 as compared to the respective vehicle (DMSO) pretreated neutrophils.

Figure 4: Effect of ActD on IL-8 mRNA expression, IL-8 protein synthesis and release by the neutrophils. Neutrophils were incubated either with the vehicle (DMSO) or ActD (5 μg/ml) for 30 minutes before the addition of PBS, fMLP (10^{-7} M), LPS (10 μg/ml) and Ang1 (10^{-9} and 10^{-8} M) for a period of 1 to 6 hours. IL-8 mRNA was quantified by RT-qPCR, whereas IL-8 protein quantification was performed as described in figures 1 and 2. For the IL-8 mRNA study, the PBS value at each time point was set to one and IL-8 mRNA fold of increase was compared to the normalized PBS at corresponding time points (A). Synthesis rate represents the quantity of IL-8 protein synthesized per hour by the neutrophil between each time point (B). IL-8 protein release is the quantity of IL-8 released at corresponding time points (C). Results were expressed as the mean ± SEM of at least three

independent experiments. Statistical comparisons were performed by analysis of variance (ANOVA) followed by a Bonferroni *t*-test. *p< 0.05, **p< 0.01 and ***p< 0.001 as compared to the respective PBS ± ActD pretreated neutrophils and †p< 0.05, ††p< 0.01 and †††p< 0.001 as compared to the respective vehicle (DMSO) pretreated neutrophils.

Figure 5: Effect of SB203580, U0126 and Triciribine on IL-8 mRNA expression, IL-8 protein synthesis and release by the neutrophils. Neutrophils were incubated either with the vehicle (DMSO), SB203580 (1 or 10 μM), U0126 (2 or 20 μM) or Triciribine (1 or 5 μM) for 30 minutes before the addition of PBS, fMLP (10^{-7} M), LPS (10 μg/ml) and Ang1 (10^{-9} and 10^{-8} M) for a period of 1 or 2 hours. IL-8 mRNA was quantified by RT-qPCR, whereas IL-8 protein quantification was performed as described in figures 1 and 2. For the IL-8 mRNA study, the PBS value after 1 hour was set to one and the modulation of IL-8 mRNA expression (fold of increase) was compared to the normalized PBS-treated neutrophils (A). Cumulative IL-8 protein represents the total quantity of IL-8 protein synthesized by the neutrophils (B), whereas IL-8 protein release is the quantity of IL-8 released at 2 hours (C). Results were expressed as the mean ± SEM of at least three independent experiments. Statistical comparisons were performed by analysis of variance (ANOVA) followed by a Bonferroni *t*-test. *p< 0.05, **p< 0.01 and ***p< 0.001 as compared to the respective PBS-treated cells and †p< 0.05, ††p< 0.01 and †††p< 0.001 as compared to the respective vehicle (DMSO) pretreated neutrophils.

Figure 6: Effect of U0126 on ERK-1/2 phosphorylation by the neutrophils. Neutrophils were incubated either with PBS, fMLP (10^{-7} M) and Ang1 (10^{-8} M) for a period of 5 to 60 minutes. The PBS value was set to one at each time point and the modulation of p-ERK-1/2 (fold of increase) was compared to the normalized PBS-treated neutrophils (A). Neutrophils were pretreated for 30 minutes with PBS, DMSO or U0126 (2 or 20 μM) before the addition of PBS, fMLP (10^{-7} M) and Ang1 (10^{-8} M)

for an additional 30 minutes. The PBS-treated neutrophils value was set to one and the modulation of p-ERK-1/2 (fold of increase) was compared to this normalized value (B). Results were expressed as the mean ± SEM of at least three independent experiments. Statistical comparisons were performed by analysis of variance (ANOVA) followed by a Bonferroni *t*-test. *p< 0.05, **p< 0.01 and ***p< 0.001 as compared to the respective PBS-treated cells and †p< 0.05, ††p< 0.01 and †††p< 0.001 as compared to the respective vehicle (DMSO) pretreated neutrophils.

Table I: Percentage of IL-8 released by neutrophils

Treatment	Cumulative IL-8 released ($ng/5x10^6$ neutrophils)	Cumulative IL-8 synthesized ($ng/5x10^6$ neutrophils)	IL-8 released (%)
PBS	0.05 ± 0.02	9.83 ± 1.18	**0.46 ± 0.08**
fMLP (10^{-7} M)	0.57 ± 0.11***	14.72 ± 1.63*	**4.15 ± 0.84****
LPS (10 µg/ml)	1.60 ± 0.46***	25.93 ± 2.05***	**5.70 ± 1.05****
Ang1 (10^{-9} M)	0.08 ± 0.03	10.41 ± 1.04	**0.78 ± 0.29**
Ang1 (10^{-8} M)	0.33 ± 0.09***	13.10 ± 1.06**	**2.45 ± 0.55****

Cumulative IL-8 released represents the IL-8 measured by ELISA from the supernatant, whereas cumulative IL-8 synthesized represents the IL-8 collected from the supernatant and inside neutrophils after the 6-hour treatment. IL-8 released (%) represents the fraction of the cumulative IL-8 released divided by the cumulative IL-8 synthesized. Data is expressed as mean ± SEM. Statistical comparisons were performed by analysis of variance (ANOVA) followed by a Bonferroni *t*-test. *$p< 0.05$, **$p< 0.01$ and ***$p< 0.001$ as compared to PBS treatment.

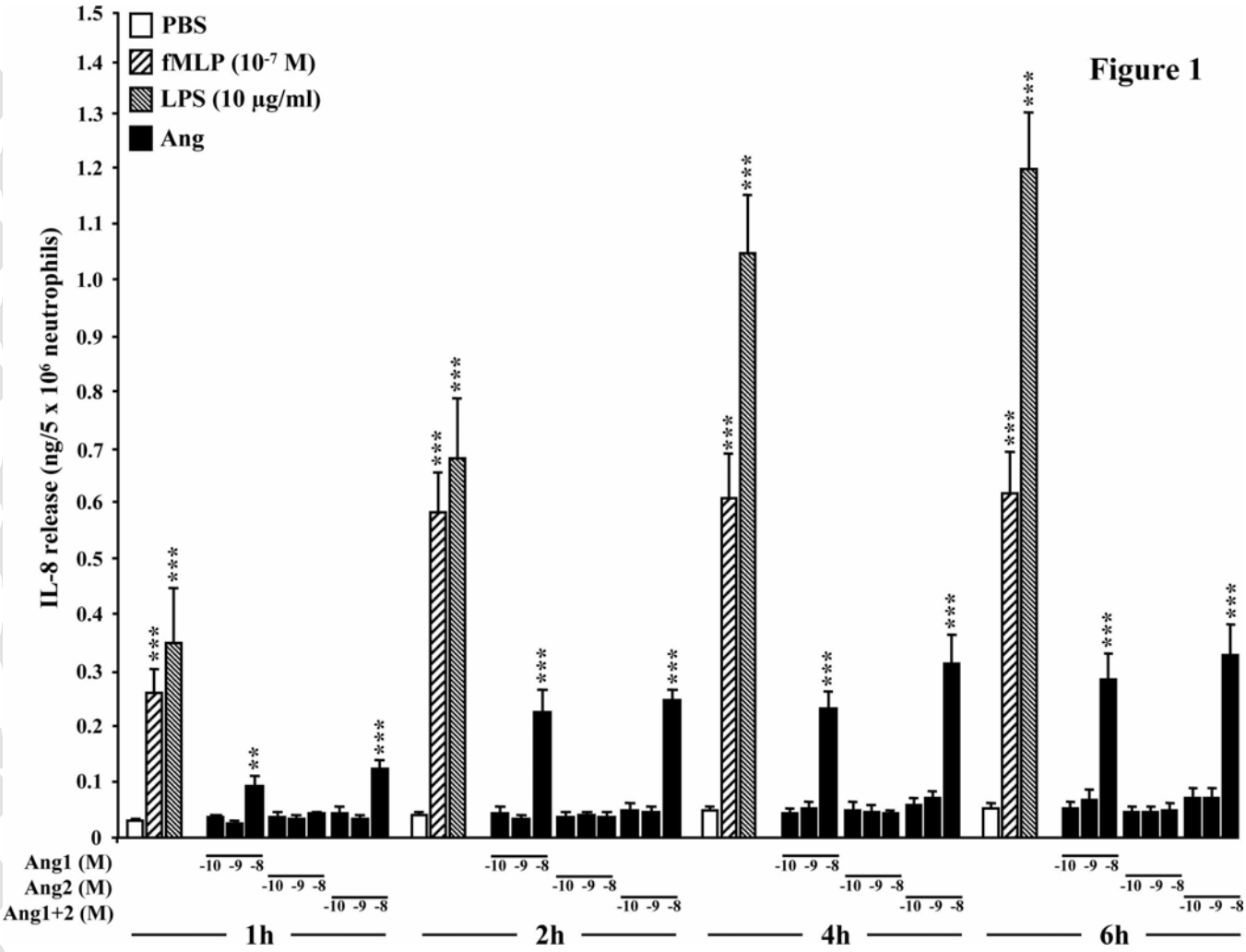
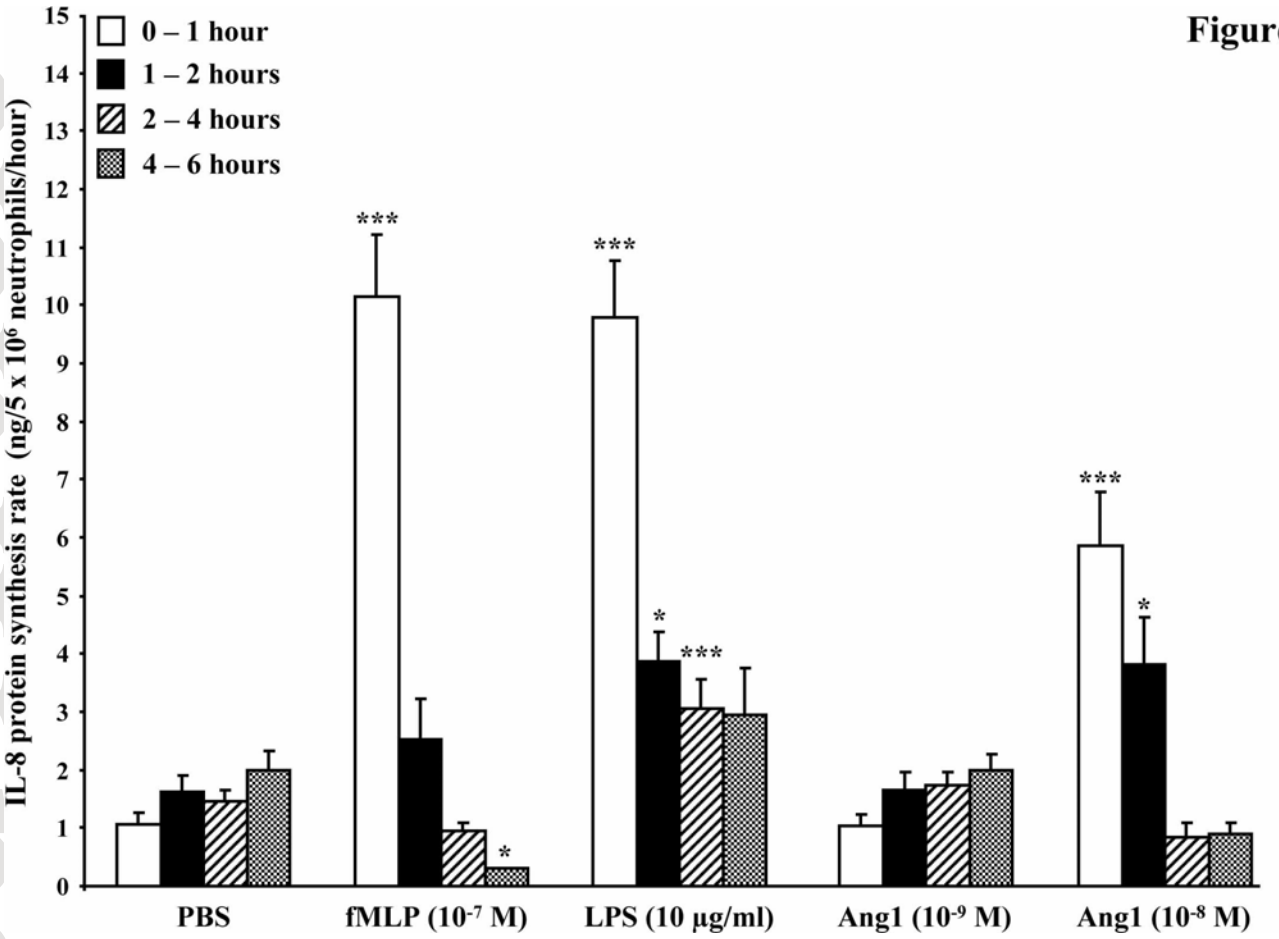


Figure 1

Figure 2A



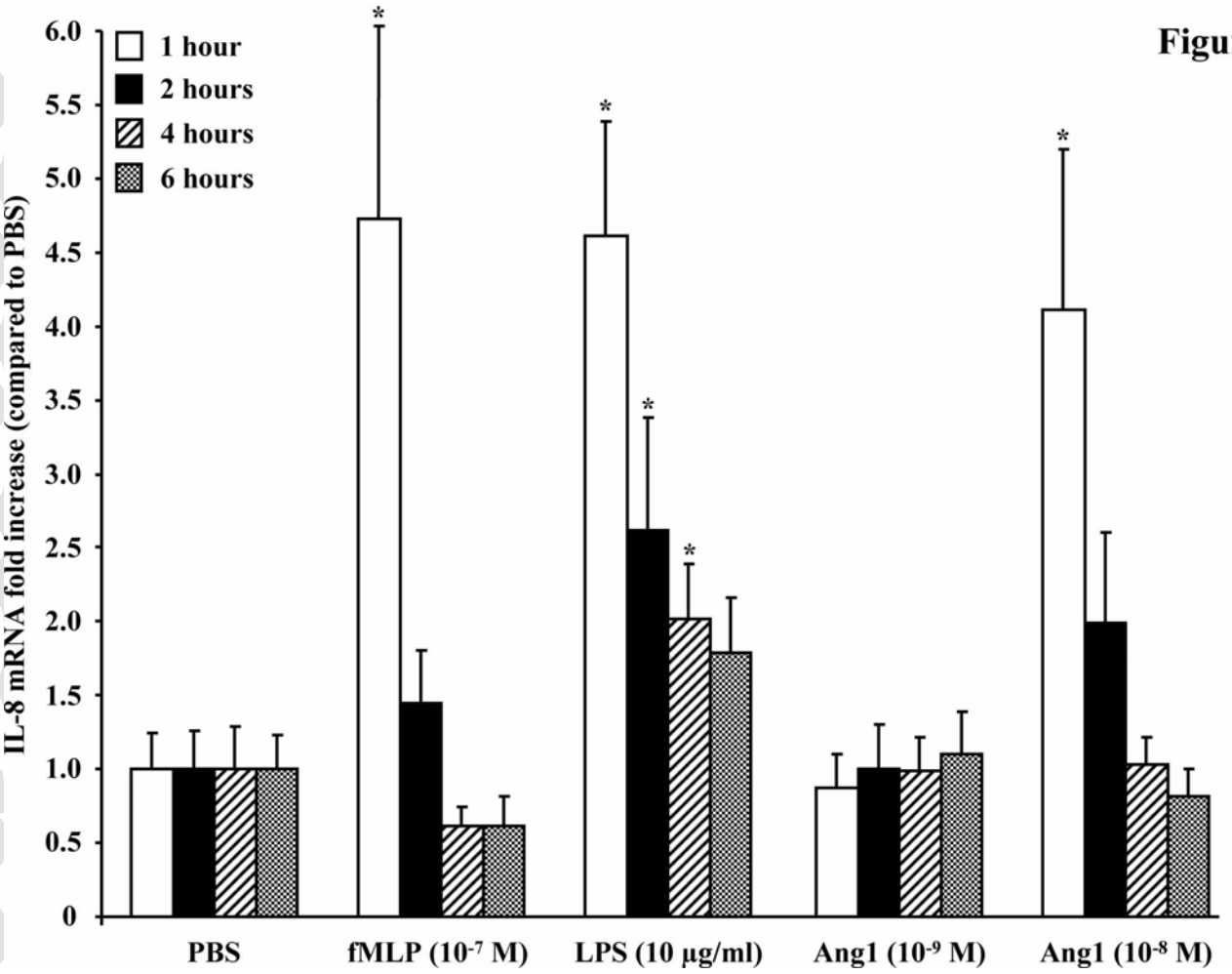
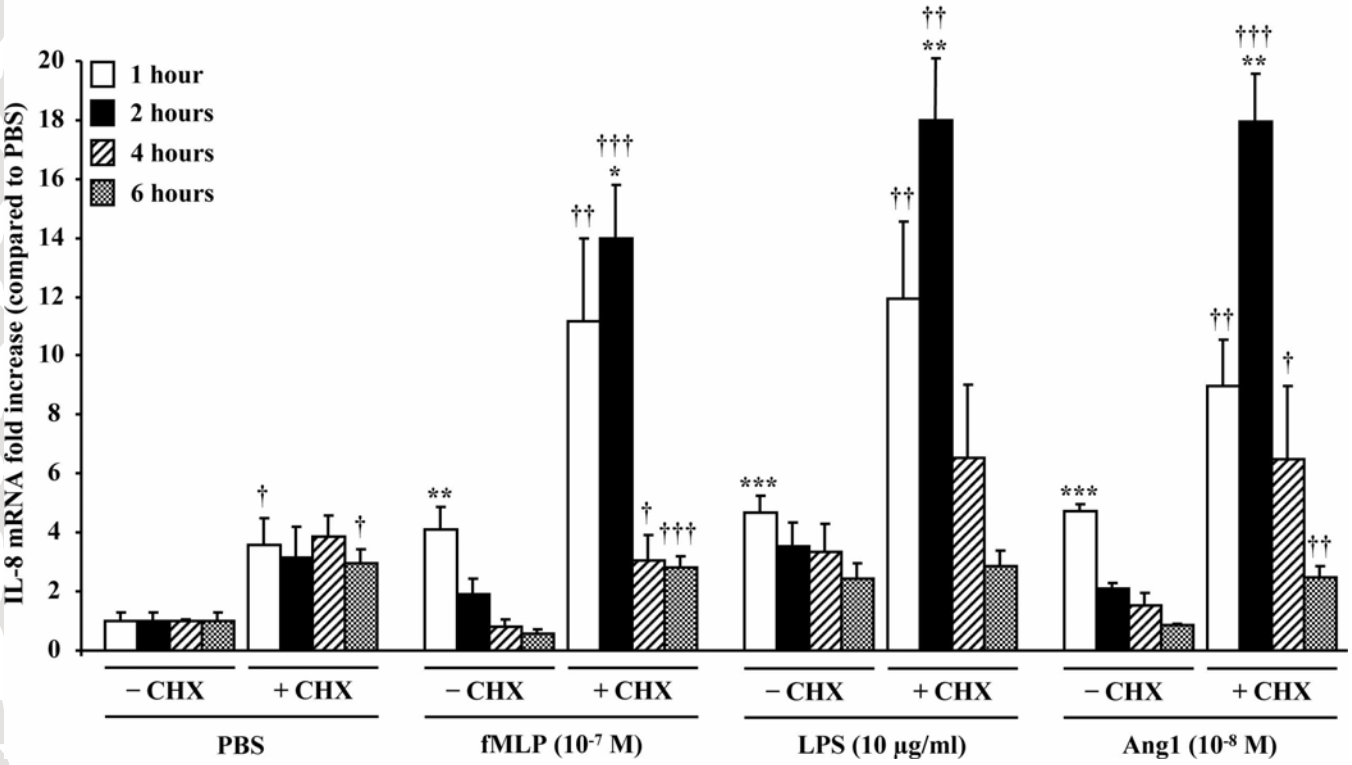


Figure 2B

Figure 3A



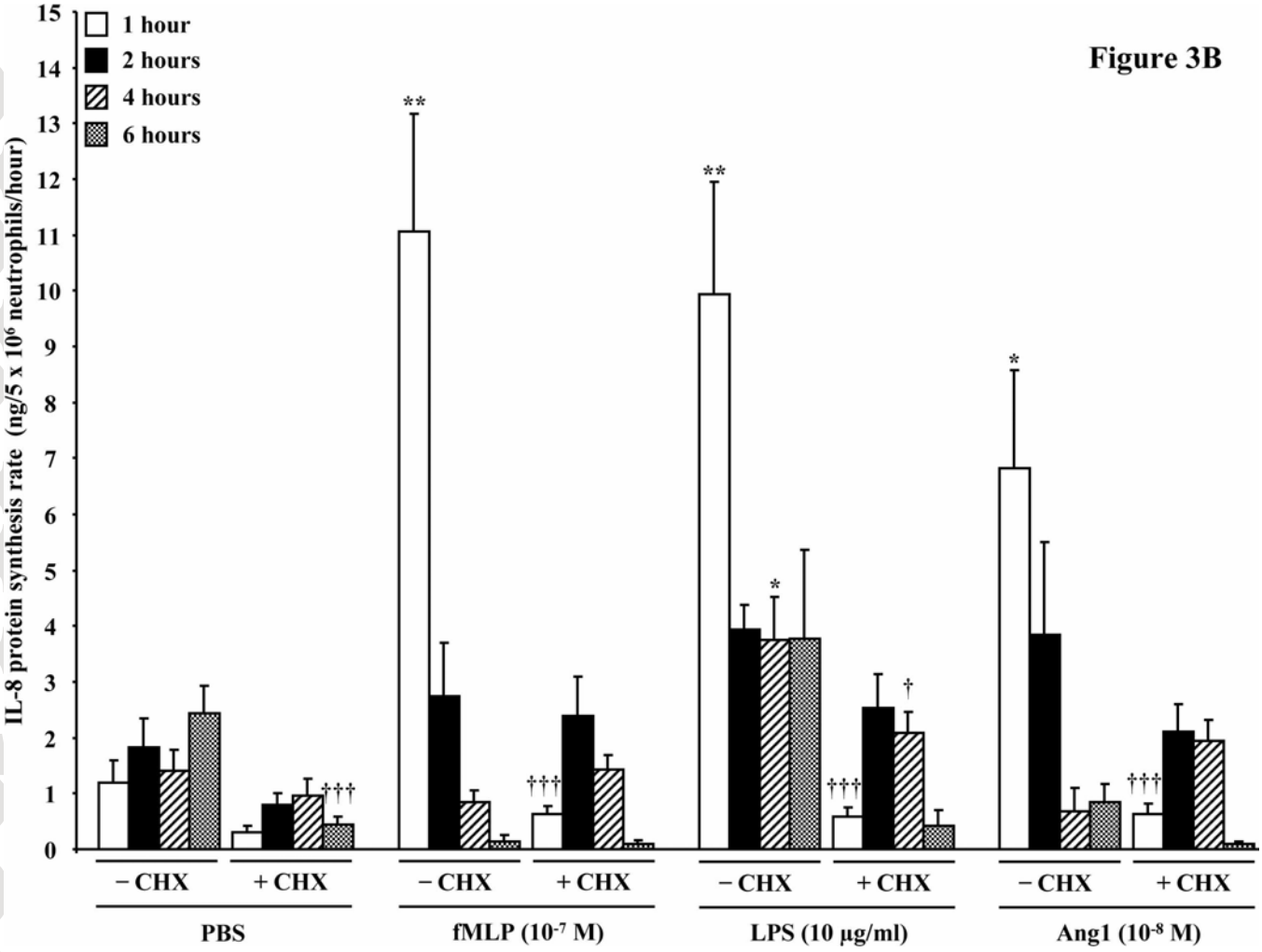


Figure 3B

Figure 3C

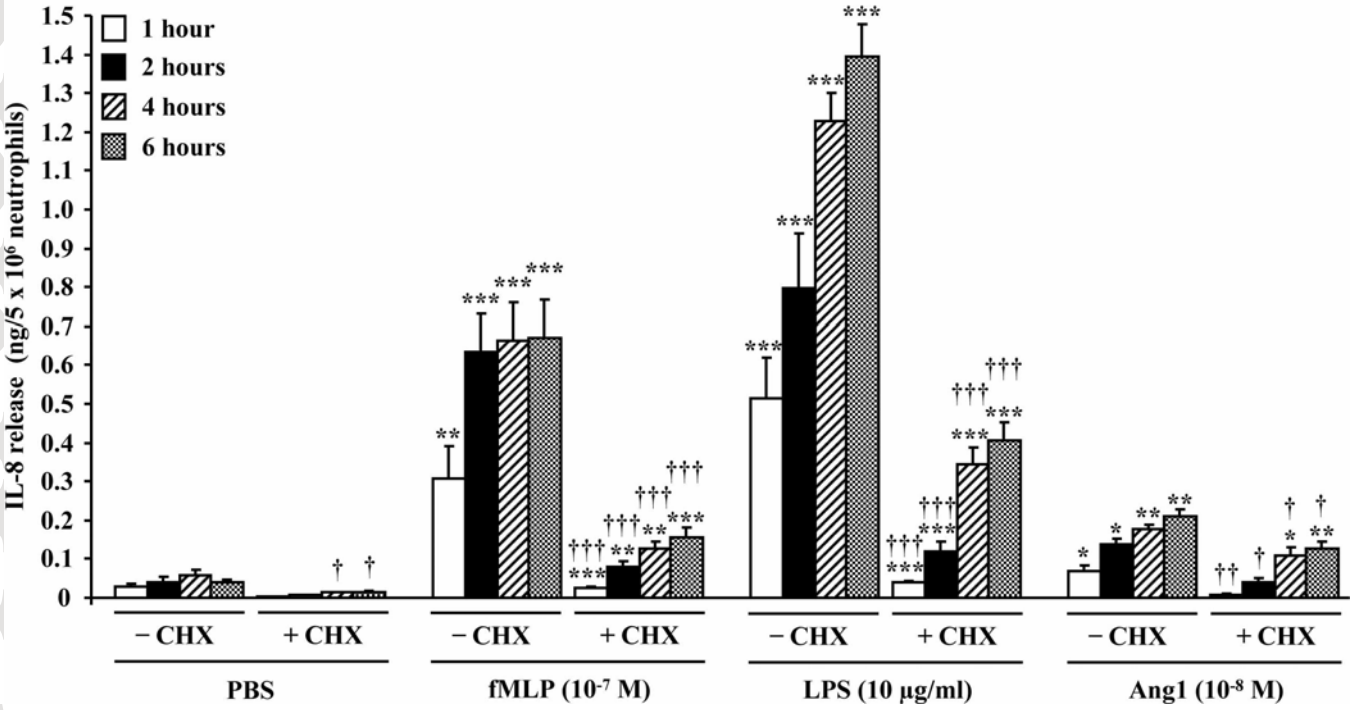
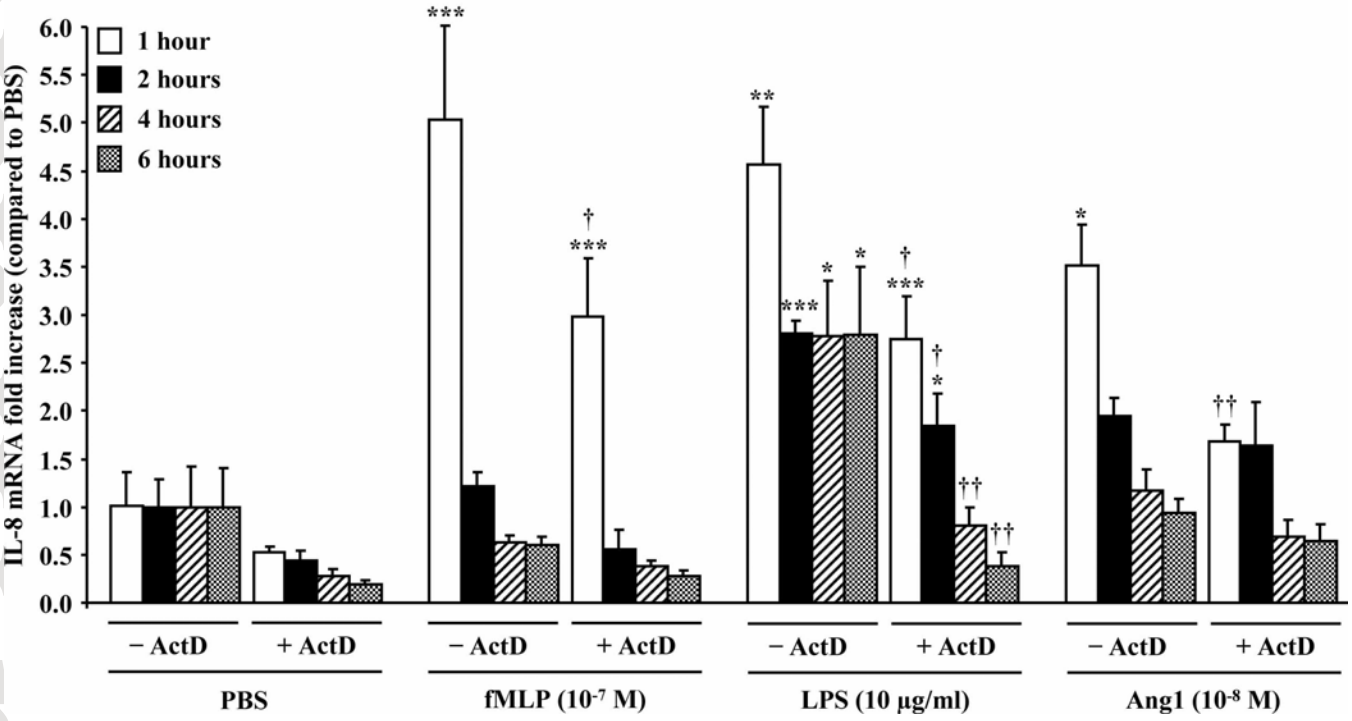


Figure 4A



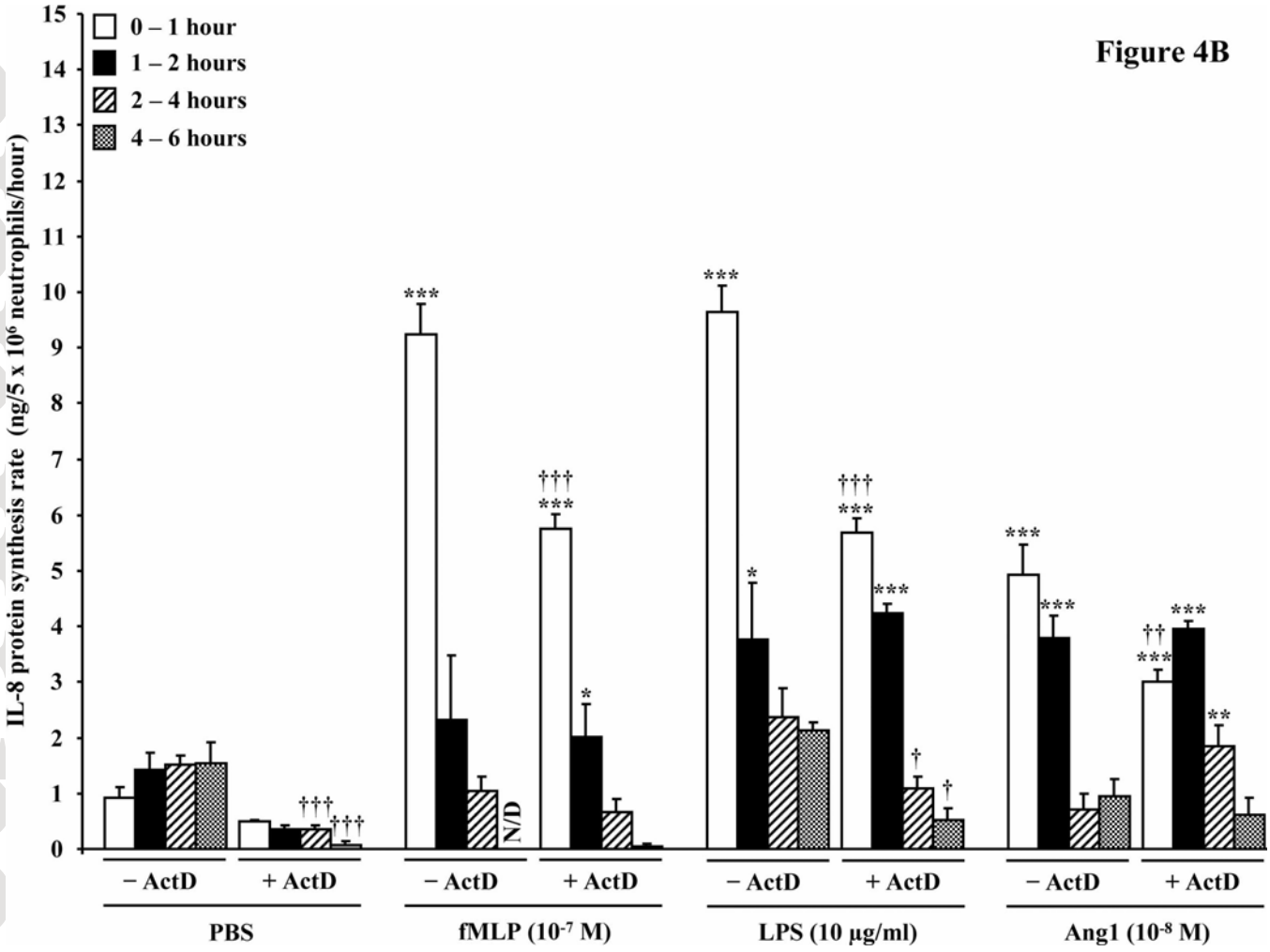


Figure 4B

Figure 4C

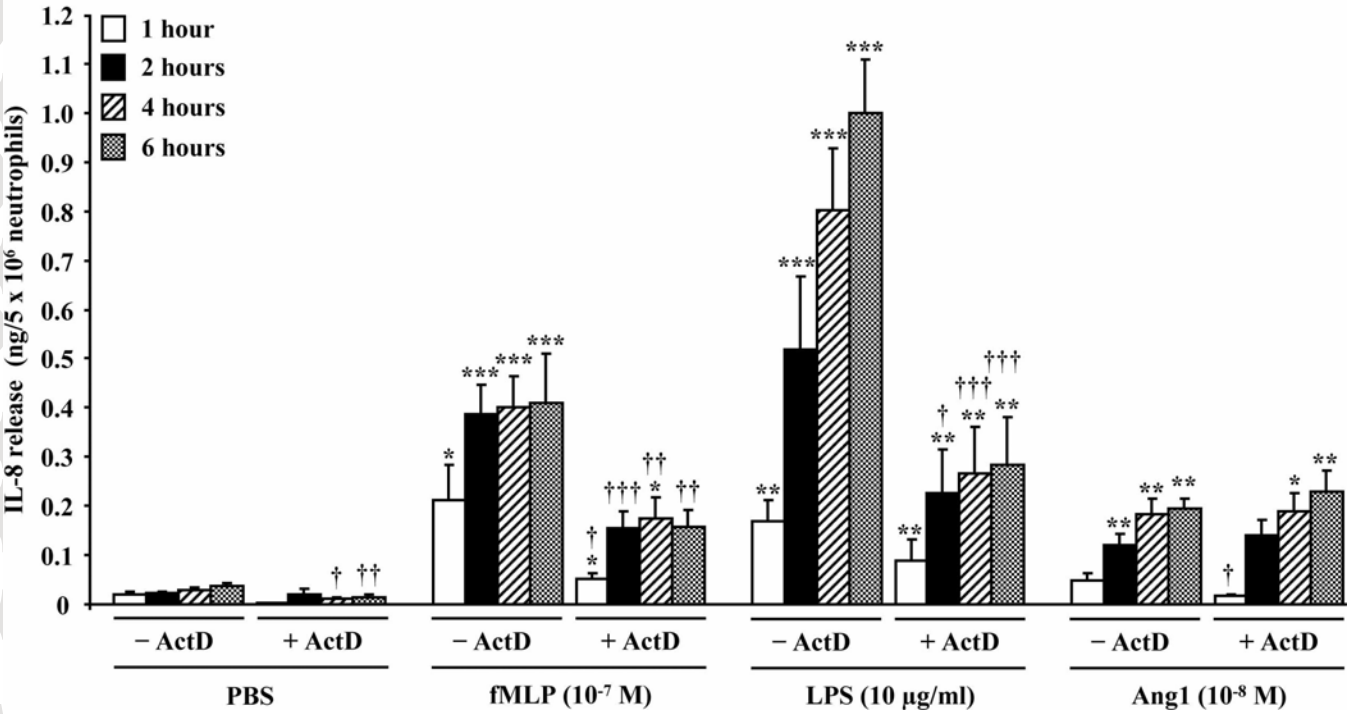


Figure 5A

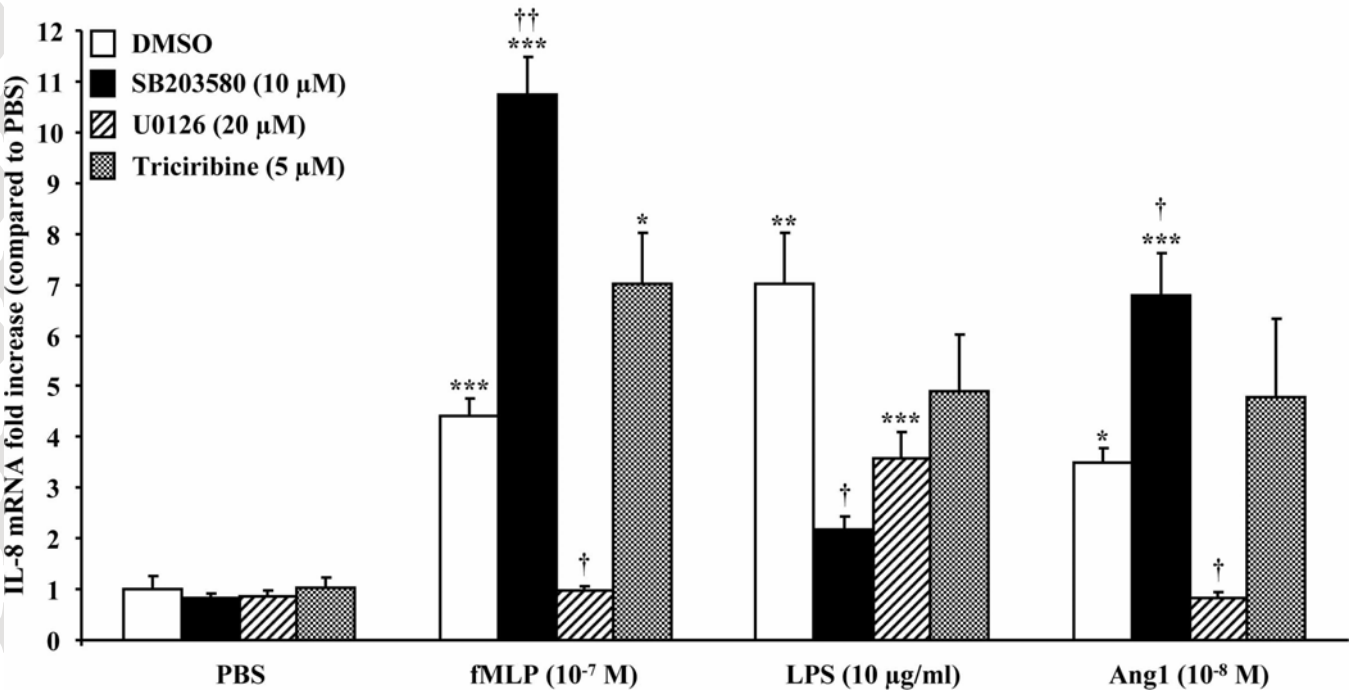


Figure 5B

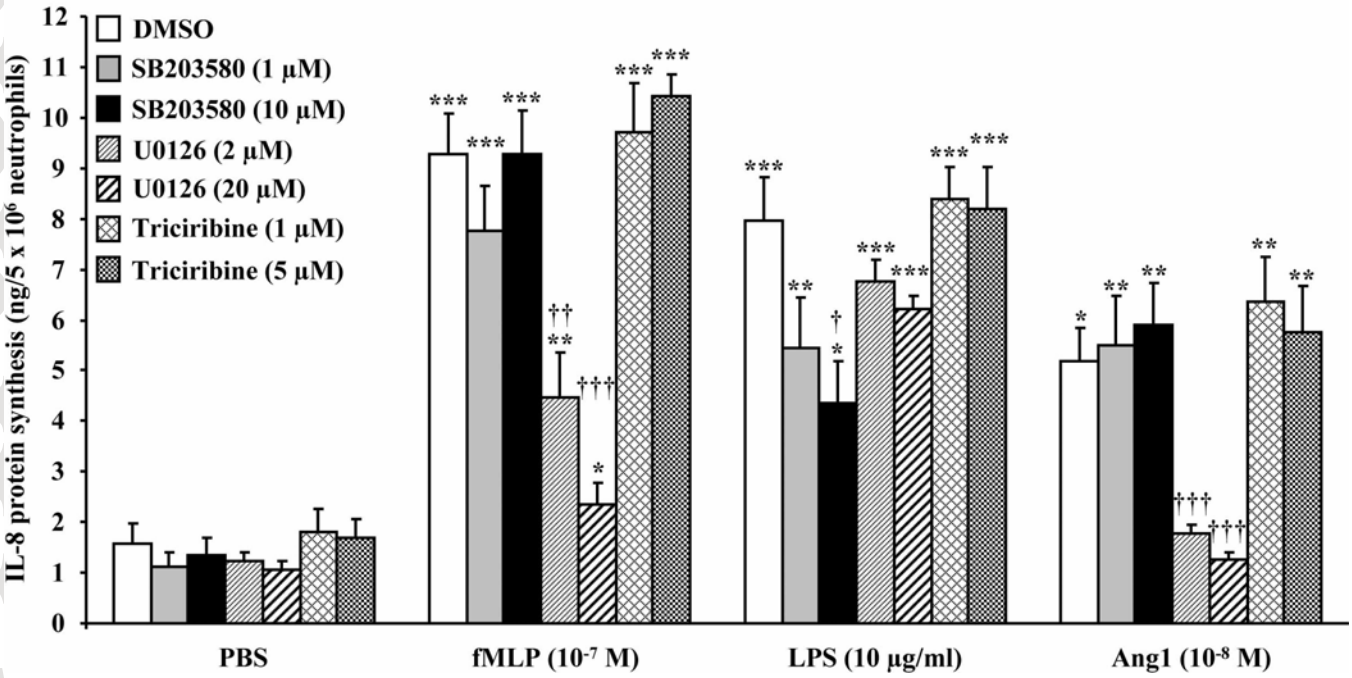


Figure 5C

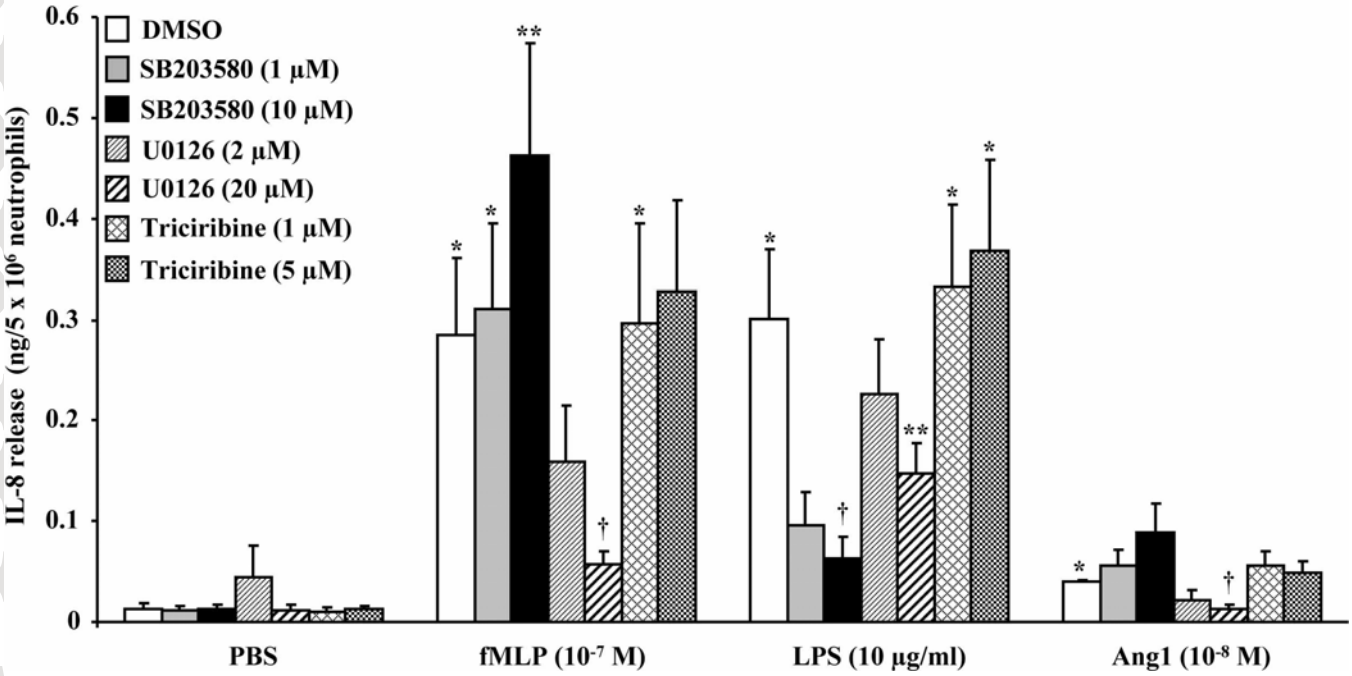


Figure 6A

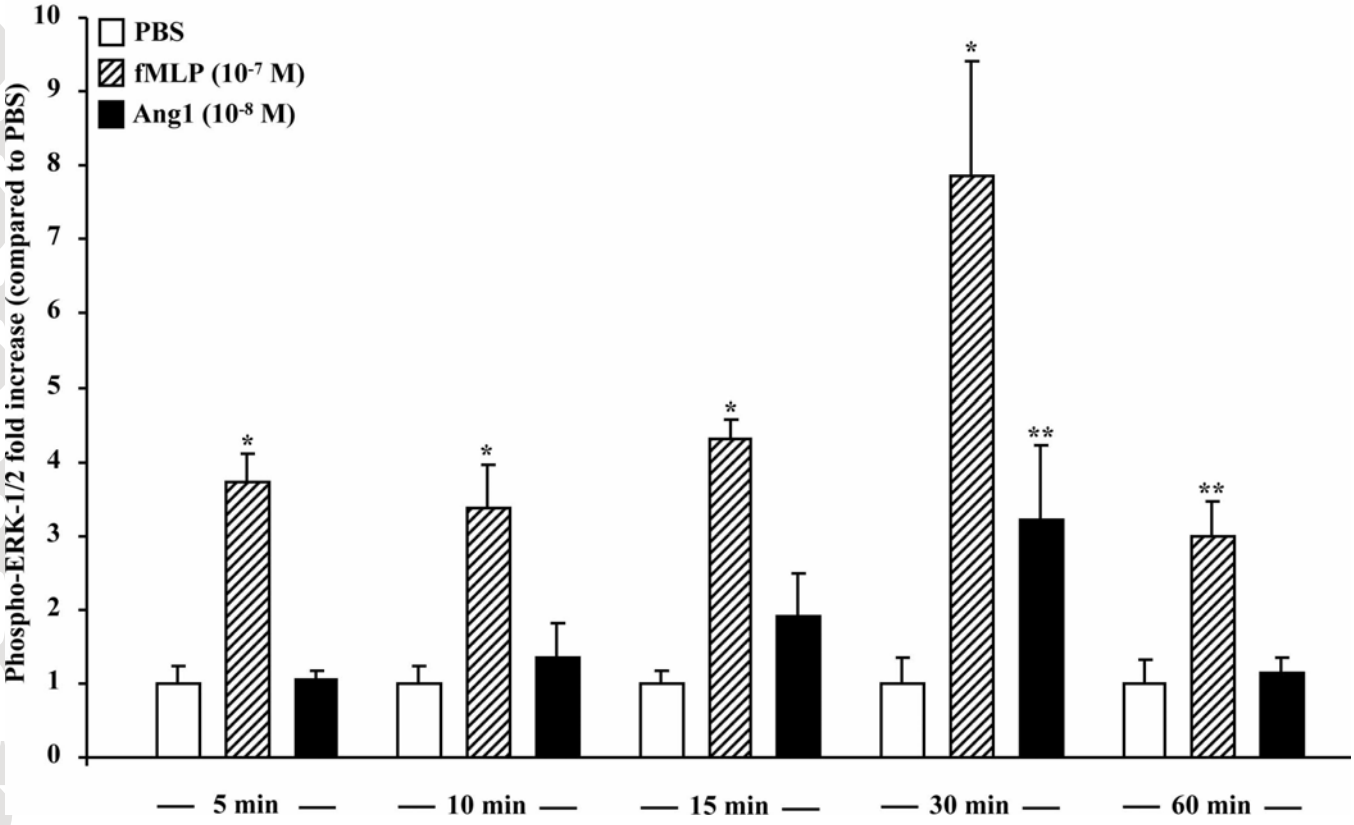


Figure 6B